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Semi-interpenetrating nanofiber scaffolds for transbuccal drug delivery

Donald Aduba Jr.
Virginia Commonwealth University

School of Engineering
Virginia Commonwealth University

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SEMI-INTERPENETRATING NANOFIBER SCAFFOLDS FOR TRANSBUCCAL DRUG DELIVERY has been approved by his committee as satisfactory completion of the thesis requirement for the degree of Master of Science in Biomedical Engineering

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Dr. Hu Yang, Ph.D., Thesis Advisor, Department of Biomedical Engineering

---

Dr. Gary L. Bowlin, Ph.D., Thesis Co-Advisor, Department of Biomedical Engineering

---

Dr. W. Andrew Yeudall, Ph.D., Thesis Co-Advisor, School of Dentistry

---

Dr. Gerald E. Miller, Ph.D., Undergraduate Chair. Department of Biomedical Engineering

---

Dr. Rosalyn Hobson Hargraves, Ph.D., Associate Dean of Graduate School of Engineering

---

Dr. J. Charles Jennett, Ph.D., Interim Dean, School of Engineering

---

Dr. Douglas F. Boudinot, Ph.D., Dean of the School of Graduate Studies

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SEMI-INTERPENETRATING NANOFIBER SCAFFOLDS FOR TRANSBUCCAL DRUG DELIVERY

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biomedical Engineering at Virginia Commonwealth University

By

Donald Aduba, Jr.

Bachelor of Science, University of Virginia, 2008

Director: Hu Yang, Ph.D., Associate Professor, Biomedical Engineering

Virginia Commonwealth University
Richmond, Virginia

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Table of Contents

List of Tables

List of Figures

Abstract

SEMI-INTERPENETRATING NANOFIBER SCAFFOLDS FOR TRANSBUCCAL MUCOSA DRUG DELIVERY

By Donald Chukwuemeka Aduba, Jr., M.S.

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Virginia Commonwealth University, 2012

Research Director: Dr. Hu Yang
Qimonda Associate Professor, Biomedical Engineering

The oral buccal mucosa is a promising absorption site for drug administration because it is permeable, highly vascularized and allows ease of administration. Although there are many platforms that have been used for drug delivery, nanofiber scaffolds as a platform for local or systemic drug delivery through the oral mucosa have not been fully explored. In this thesis, we fabricated a biocompatible electrospun gelatin nanofiber scaffold for local drug delivery at the oral mucosa. To stabilize the electrospun gelatin nanofibers and allow non-invasive incorporation of therapeutics into the scaffold, photo-reactive polyethylene-glycol (PEG)-diacrylate was employed to crosslink the scaffold to form semi-interpenetrating networks (sIPNs). The crosslinking parameters including concentration of PEG-diacrylate, amount of photoinitiator, and crosslinking incubation time of the scaffold were systematically investigated. The resulting scaffolds were characterized in terms of their morphology, tensile properties, porosity, swelling and degradation. The results confirmed that gelatin electrospun nanofiber scaffolds after being photo-crosslinked with PEG-diacrylate retain fiber morphology and show improved structural stability and mechanical properties. The mucoadhesiveness of the sIPN nanofiber scaffold was confirmed. Nystatin, a drug to treat fungal infections such as candidiasis was loaded to the sIPN nanofiber scaffold. Its release kinetics was also studied.

CHAPTER 1: INTRODUCTION

Oral candidiasis has become a growing affliction within the oral cavity primarily due to the human immunodeficiency virus (HIV). Oral candidiasis manifests itself in fungal infections of the oral cavity due to poor dental hygiene or within the denture wearing elderly patients. At its inception, candidiasis is a common pathological symptom that stems from the infection of any of the Candida species such as *Candida albicans*.[1,2] From a clinical perspective, oral candidiasis is a source of oral discomfort, pain, bad taste sensation and loss of appetite. Candidiasis, commonly called oral thrush, is shown as white clusters of bacteria lining the cheeks, tongue and gum lining of the oral mucosa. This infection can spread into the esophagus causing more threatening secondary infections.[3]

There are a variety of antifungal drugs available to treat candidiasis. These drugs are commonly administered topically or systemically ranging from amphotericin lozenges and oral suspensions to azole-group antimycotics in the form of creams, tablets and capsules.[3,4] The recent exploration of using polymeric biomaterials for treatment of oral candidiasis has led to the emergence of many biotherapeutics on the market. Although nanofiber scaffolds have been widely used in the fields of tissue engineering and wound healing, their utility in drug delivery, particularly local drug delivery to treat oral diseases, has been limited. In this study we fabricated a multidimensional gelatin nanofiber scaffold crosslinked with PEG-diacrylate. The use of natural polymers such as gelatin enables the scaffold to have widely tunable mechanical properties while ensuring biocompatibility with the surrounding cells and tissues within the oral mucosa. This scaffold was used to encapsulate nystatin, an anti-fungal drug for potential local treatment of candidiasis. This scaffold also possesses mucoadhesive properties to allow

attachment to the oral mucosal tissue and locally deliver nystatin to the regions affected by candidiasis. The formulation is designed for time-sensitive degradation to target a large drug payload released within six hours to ensure patient compliance.

CHAPTER 2: BACKGROUND

2.1 Anatomy and physiology of the oral mucosa

The oral cavity comprises the lips, buccal, tongue, hard palate, soft palate and floor of the mouth. The lining of the oral cavity is referred to as the oral mucosa, which consists of the buccal, sublingual, gingival, palatal and labial mucosa. The mucosal tissues in the cheeks (buccal), the floor of the mouth (sublingual) and the ventral surface of the tongue account for approximately 60% of the oral mucosal surface area. In the context of drug delivery, the buccal and sublingual tissues are the primary absorption sites because they are more permeable than the tissues in the other regions of the mouth.[5]

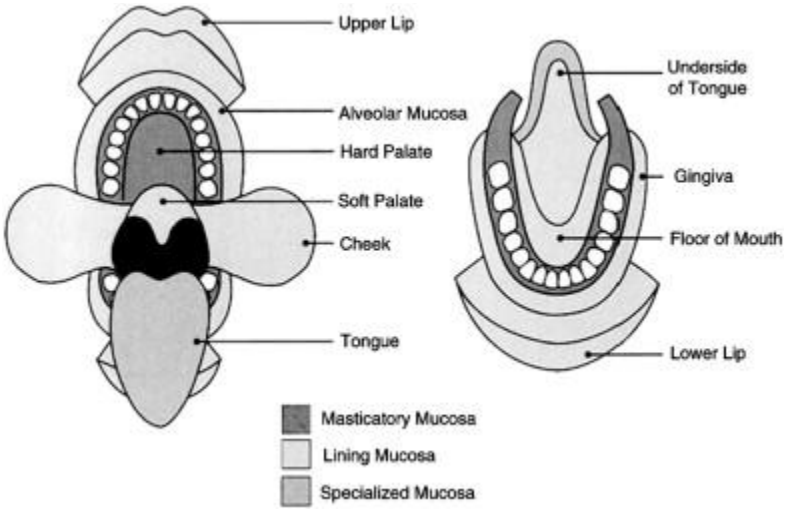

Figure 2.1: Anatomy of the oral cavity. (Adapted from Squier [6])
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The oral mucosa is composed of three tissue layers. The outermost layer is a stratified epithelium. Beneath the epithelium is the lamina propria followed by the submucosa, the inner most layer of the oral mucosa.[7] The degree of keratinization and thickness of the buccal mucosa varies within different regions of the oral cavity. These two properties, in turn, influence the permeability of solutes across the oral mucosa.

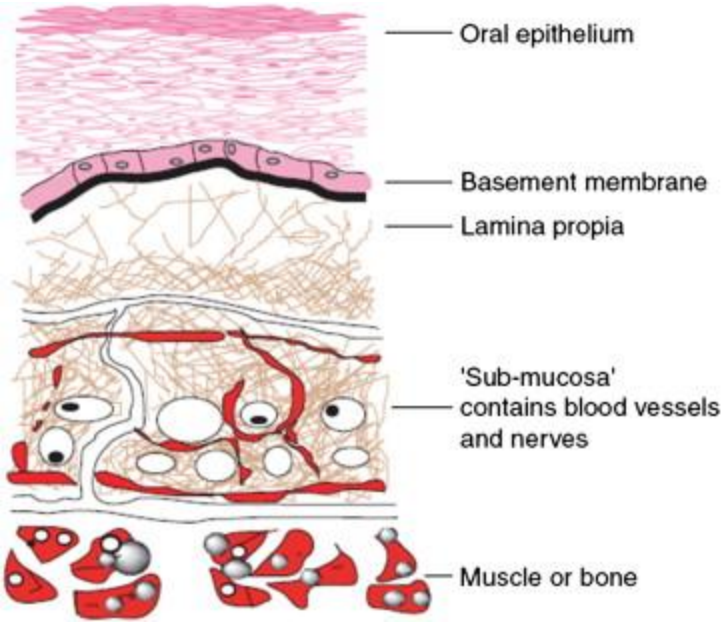

Figure 2.2: Anatomy of oral mucosa. (Adapted from Smart, J.D. [8])
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The primary function of the oral epithelium is to protect the underlying tissue.[9] It is the first layer that drugs must pass through. Similar to other epithelia in the body, it has a common mitotically active basal cell layer that advances through many differentiating intermediate layers to the superficial layers. The epithelium of the buccal mucosa is about 40-50 cell layers thick. These epithelial cells increase in size and become flatter as they progress from the basal layers to the superficial layers.[10] The buccal mucosa epithelium takes approximately 5-6 days to turnover its cells. The composition is dependent on its location in the oral cavity. In mammals, the gingivae and hard palate areas are more likely to be keratinized while the mucosa of the soft palate, sublingual (underside of tongue) and buccal regions are non-keratinized.[11] Keratinized buccal mucosa epithelia contain neutral (no charge) lipids such as ceramides and acylceramides, making it impermeable to water and water soluble drugs. Non-keratinized epithelia do not contain ceramides or acylceramides. They contain relatively neutral but polar lipids which are

mainly cholesterol sulfate and glucosyl ceramides.[12] These types of epithelia are more permeable to water in comparison to keratinized epithelia.[11]

2.2 Permeability and environment of oral mucosa

2.2.1 Permeability of oral mucosa

The oral mucosa is a moderately leaky epithelium between the epidermis and intestinal mucosa. Mucosa surface absorption is efficient compared to skin because the mucosa does not have the stratum corneum epidermis, a significant permeation barrier of the skin.[5] The permeability of the buccal mucosa is 4-4000 times greater than the skin.[13] There are significant differences in permeability between different regions of the oral cavity because of the heterogeneous structures and functions of the oral mucosa. Generally, the permeability of the oral mucosa decrease in the order of sublingual, buccal, and palatal.[11]

Table 2.1: Physical characteristics of oral mucosal tissue.

Tissue	Structure	Permeability	Blood Flow (ml/min/100g tissue)	References
Buccal	Non-Keratinized	Intermediate	20.3	[14], [16]
Sublingual	Non-Keratinized	Poor	12.2	[14], [16]
Gingival	Keratinized	Intermediate	19.5	[14], [16]
Palatal	Keratinized	Very Good	7.0	[14], [16]

It is believed that the permeability barrier of the oral mucosa is a result of intercellular material derived from membrane coating granules (MCGs).[17] MCGs are formed while cells are undergoing differentiation. They form at the apical cell surfaces where they fuse with the plasma membrane. After fusion, their contents are discharged into the upper one-third of the epithelium. This permeability barrier is at the outermost 200 μm of the superficial layer of the oral mucosa epithelium.

The permeability of a barrier can be evaluated by utilizing tracers.[18,19] When applied to the outer surface of the epithelium, these tracers penetrate through the outermost two layers of cells. On the submucosal surface, the tracers permeate to the surface of the outermost cell layers of the epithelium. According to permeability studies, flattened surface cell layers are the main barrier to permeation. Iso-diametric cell layers are relatively permeable due to their evenly distributed dimensions.[19] In both keratinized and non-keratinized epithelial layers, permeation does not occur where MCGs are adjacent to the superficial plasma membranes of the epithelial cells.[19]

The structures of the MCGs in keratinized and non-keratinized epithelia are different.[12] The MCGs of keratinized epithelium are composed of lamellar lipid stacks, whereas the MCGs of non-keratinized epithelium contain non-lamellar lipid stacks. More specifically, the MCGs of keratinized epithelia are composed of sphingomyelin, glucosylceramides, ceramides, and other non-polar lipids. The MCGs of non-keratinized epithelia are made of cholesterol esters, cholesterol, and glycosphingolipids.[12] Outside of MCGs, the oral mucosa's basement membrane may present resistance to permeation but its outer epithelium is still considered to be a rate limiting step to mucosal penetration. This is because the structure of the basement membrane is not dense enough to prevent the entry of relatively large molecules such as horseradish peroxidase tracers commonly used in permeation studies.[10]

2.2.2 Environment of oral mucosa

The oral epithelia cells are covered by mucus made of proteins and carbohydrates. Mucus has a thickness ranging from 40 μm to 300 μm and a molecular mass that ranges from 0.5 to over 20 mega daltons.[20,21] These biochemical complexes can be either free of association with or bound to certain regions on the cell surfaces. The matrix of these components plays a role in cell

to cell adhesion interactions, acting as a lubricant to allow cells to move relative to one another.[22] Mucus also plays a role in bioadhesion of mucoadhesive drug delivery systems.[23] In the oral mucosa, mucus is secreted by major and minor salivary glands as part of saliva.[22,24] Up to 70% of mucin in saliva is produced by the minor salivary glands.[22,24] At physiological pH of 7.4, the mucus network carries a negative charge due to sialic acid and sulfate residues which may play a role in mucoadhesion. At physiological pH, mucus can form a strongly cohesive gel structure that binds to the epithelial cell surface as a gelatinous layer.[7] Mucus molecules are able to join together to make polymers or form an extended three-dimensional network.[5]

Another feature of the oral cavity environment is the presence of saliva produced by the salivary glands. Saliva is a fluid present in the oral cavity protecting the soft tissues from abrasion. It also allows for the continuous mineralization of tooth enamel after eruption and helps with remineralization of the enamel in the early stages of tooth decay.[25] Saliva is an aqueous fluid containing 1% organic and inorganic materials. The salivary pH ranges from 5.5 to 7. At high flow rates, the sodium and bicarbonate concentrations increase, resulting in a spike of the salivary pH. Daily salivary volume ranges from 0.5 to 2 liters, needed to hydrate oral mucosal dosage forms.[10]

2.3 Oral mucosa drug absorption

2.3.1 Principles of drug absorption via the oral mucosa

The oral mucosa has been scrutinized as an absorption site for drug administration because its anatomical properties allow for localized and sustained release of therapeutic over an extended period of time. The surface area of the oral mucosa is relatively small compared to the gastrointestinal tract and skin. Nonetheless, the oral mucosa is highly vascularized, allowing drug to diffuse into the oral mucosa membranes and get into the systemic circulation. Drug

administration via the oral mucosa bypasses the gastrointestinal tract and first-pass metabolism in the liver.[26] Because of the ampiphilic properties of the epithelial cell layer, the oral mucosa represents a tissue barrier for drug absorption. The ampiphilicity of the epithelial cell layer can regulate drug absorption via diffusion, ion transport, endocytosis and electroporation mechanisms. The absorption of drug molecules across the cell layer can be affected by size or polarity of the drug. Enzymes present at the barrier can cause rapid degradation of peptides and proteins, hence limiting their transport across the oral mucosa. Several drug design approaches have been developed to circumvent limitations of oral mucosa delivery.[5] An in-depth understanding of the oral mucosa absorption mechanisms is necessary to design effective drug delivery vehicles to overcome permeation barriers of the mucosa epithelia.

2.3.2 Drug absorption mechanisms across the oral mucosa

Drug absorption via the oral mucosa is a passive diffusion process that can be described by Fick's first law,

$$J = -D\frac{\delta c}{\delta x} \quad (1)$$

The diffusion coefficient (D), concentration (C) and thickness (x) of the tissue are parameters that influence drug absorption over the oral mucosa's surface area gradient. Other important parameters like surface area, duration of drug delivery and concentration should be taken into account as well. The amount of drug absorbed is dependent on several factors: drug concentration, vehicle of drug delivery, mucosal contact time, venous pH of the absorption site, size of the drug molecule, and relative lipid solubility.[27]

Drugs can permeate through the epithelial membranes of the buccal mucosa via transcellular or paracellular routes.

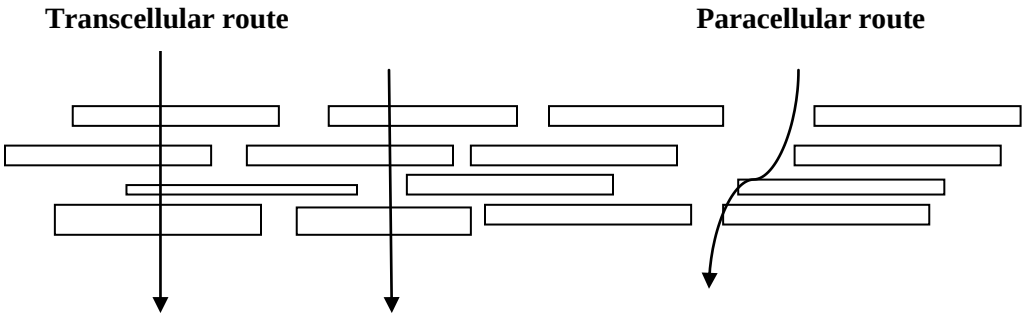


Figure 2.3: Graphical representation of different routes of drug permeation.
(Adapted from Patel, et al. [28])

As illustrated in Figure 2.3, paracellular transport is the movement of molecules around or between cells while transcellular transport is the movement of molecules through cells. Drug molecules can use these two routes of transport simultaneously although one route is preferred over the other depending on the physicochemical properties of the molecules. In paracellular transport, the spaces between cells and their cell cytoplasm are hydrophilic in nature, inhibiting the transport of lipophilic molecules. As for transcellular transport, the cell membrane's lipophilic nature would inhibit hydrophilic solutes to diffuse through the epithelial cell layer of the oral mucosa due to the solutes' low partition coefficient.[10]

2.3.3. Enhancers of oral mucosa drug absorption

Penetration enhancers can enhance drug permeation by changing the properties of the mucosa including increasing cell membrane fluidity, extracting the structural intercellular or intracellular lipids, altering cellular proteins, or altering the mucus structure and rheology.[29,30,31] Chemical enhancers could be added to a pharmaceutical formulation alone or in combination to increase the permeation without causing damage to the mucosa. Permeation enhancement efficiency by enhancers also depends on the physicochemical properties of the drug, the

administration site and the nature of the formulation. Various chemicals have been explored as permeation enhancers. Among these chemicals are chelators, surfactants, bile salts, fatty acids and non-surfactants.[5] Lately, chitosan and its derivatives have been extensively used to enhance permeation across either monostratified or pluristratified epithelia. Chitosan also improves permeation of large molecular weight hydrophilic molecules across the mucosa.[32]

Drug absorption at the oral mucosa can also be enhanced by electrophoresis, electro-osmosis and electroporation. During electrophoresis, electrical fields applied to the mucosal epithelium reduce the density of the lipids in the intercellular domain, enabling drugs to penetrate through the layer.[5] Electrical enhancement for drug permeation is most efficient for water soluble, ionized compounds. Electro-osmosis facilitates drug transport by using the inherent negative charges possessed in human tissues. These negative charges bind to mobile, positive counter ions, forming an electrically charged double layer in the tissue capillaries. When an electrical field is applied across the tissue, a net flow of water is generated through the tissue in conjunction with the solvated counter ion. In electroporation, high potential (20-100 V) pulses are applied across the tissue. The electrorestriction forces create temporary perforations or microchannels in the tissue. These channels can serve as a drug transport route and are closed within a few minutes without inducing any permanent damages to the tissue.[29,32]

2.4 Oral mucosa drug delivery systems

2.4.1. Factors of successful drug delivery within oral mucosa

Drugs in systemic circulation typically have three distinct profiles (Figure 2.4): i) rapid drug release for immediate and quick action, ii) pulsatile release with rapid appearance of drug into systemic circulation and subsequent maintenance of drug concentration within therapeutic profile or iii) controlled release over an extended period of time.[28]

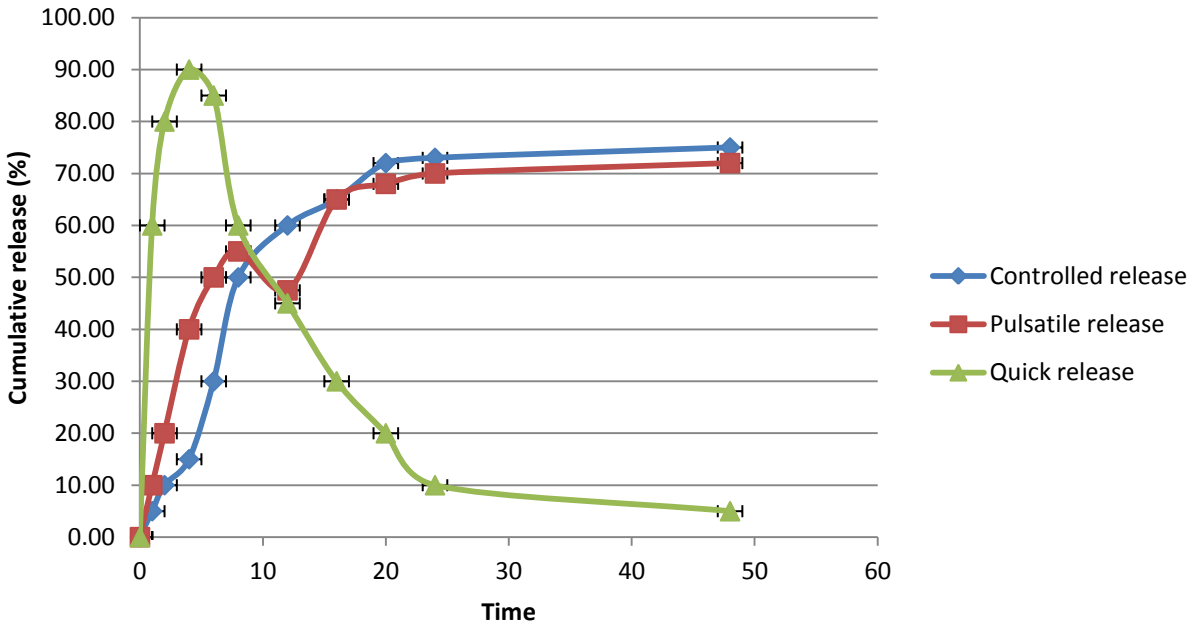


Figure 2.4: Schematic representation of different types of drug delivery release systems.
(Adapted from Patel, et al. [28])

Drug delivery vehicles for the oral mucosa should be non-toxic, non-irritable, and non-immunogenic. Typically, drug release from a polymeric material takes place either through diffusion, polymer degradation or a combination of both. Polymer degradation can be done via hydrolysis, enzymes, bulk erosion or surface erosion.[33,34] To achieve efficient drug transport across the oral mucosa, delivery vehicles or dosage forms should be tissue adhesive. The adhesiveness should be high enough to allow the formulation to rapidly attach to the mucosal surface and maintain a long residence time. Quick adhesion of the system at the target site can be achieved through "bioadhesion promoters" that use tethered polymers. Residence time is important because long residence time allows more drugs to be released at the target site. The bioadhesion of the drug delivery material should be minimally influenced by environmental factors such as the pH of the oral mucosa.

2.4.2. Oral mucosa dosage forms

The goal of oral mucosal drug delivery is not only to achieve therapeutic effectiveness but to enhance the patient's comfort level during buccal administration. Various oral mucosa drug delivery vehicles have been developed as dosage forms including solutions, tablets (lyophilized and bioadhesive), chewing gum, solution sprays, laminated systems, patches, hydrogels, adhesive films, hollow fibers, microspheres, etc.[14] It is not uncommon to utilize a combination of these dosage forms to maximize clinical efficacy while enhancing patient comfort during drug administration.

Solution forms are designed to coat the mucosa for the treatment of local disorders such as motility dysfunction and fungal infections. Sodium alginate suspension is an example of a bioadhesive liquid used to prevent reflux and deliver therapeutic agents to treat the damaged mucosa.[35,36] Tablets are in the form of solid lozenges. Examples include nitroglycerin sublingual tablets, fentanyl lozenges on a handle and prochlorperazine buccal tablets. They are easy for patients to use. The drug within the tablet is released locally to the entire oral mucosa but the tablet formulation in the oral mucosa has a short residence time. Saliva carrying the dissolved drug from the tablet often flows to the esophagus and gastrointestinal tract, resulting in loss of bioavailability of the drug.[5]

Chewing gum is a relatively new dosage form for oral mucosa drug delivery. The advantages of chewing gum include a better control over drug release and the potential to reduce variability in drug release and retention times. However, chewing gum is an open system that does not interface with the oral mucosa tissue layer. As a result, chewing gum may not be a viable vehicle for localized drug release. [5]

Adhesive patches have also been developed to avoid some drawbacks of other dosage forms. Oral mucosa delivery patches have unique characteristics including rapid onset of drug, sustained drug release and rapid decline in the drug concentration once the patch is removed. They help maintain an intimate and prolonged contact with the oral mucosa, allowing a longer duration for drug absorption. A disadvantage of patches is that they only cover a small mucosal area and their adhesive backings have to be removed manually, raising patient compliance issues.[5]

Particulate drug delivery systems such as microparticles and nanoparticles often exhibit improved performance.[37] Size, chemistry and the shape of the nanoparticles can influence particle velocity, diffusion and adhesion to the mucus surface in a complex manner. These immobilized carriers due to their relatively small size show a prolonged gastrointestinal residence time after diffusing into the mucous gel layer such as stomach lining.[38]

2.5 Applicable drugs used for delivery in oral mucosa

2.5.1 Clinical treatments in oral mucosa

Drug forms applied to the oral mucosa are generally efficient for local and systemic drug delivery. Some commercially available drugs and dosage forms used for the oral mucosa are listed in Table 2.2.

Table 2.2: Commercially available drugs for oral mucosa. (Modified from Christina, et al. [39])

Drug	Dosage Form	Type of Release	Product Name	Manufacturer
Fentanyl citrate	Lozenge	Quick	Actiq	Cephalon
	Tablet	Quick	Fentora	Cephalon
	Film	Quick	Onsolis	Meda Pharmaceutical Inc.
Buprenorphine HCl	Tablet	Quick	Subutex	Reckitt Benckiser
Buprenorphine HCL and naloxone HCl	Tablet	Quick	Suboxane	Reckitt Benckiser
Proclorperazine	Tablet	Controlled	Buccastem	Reckitt Benckiser
Testosterone	Tablet	Controlled	Striant SR	Columbia Pharmaceuticals
Nitroglycerine	Tablet	Quick	Nitrostat	W Lambert-P Davis-Pfizer Pharmaceuticals
	Spray			
Glyceryl trinitrate	Spray	Quick	Nitromist	NovaDel
Zolpidem	Spray	Quick	Zolpimist	Forest Laboratories
	Tablet	Quick	Suscard	
Nicotine	Chewing gum	Quick	Nicotinelle	Novartis Consumer Health
	Lozenge			
Miconazole	Tablet	Quick	Loramyc	BioAlliance Pharma SA
Cannabis-derived	Spray	Quick	Sativex	GW Pharmaceuticals, PLC
Insulin	Spray	Quick	Oral-lyn	Generex Biotechnology
Nystatin	Tablet	Quick	Bio-statin	Geneza Pharmaceuticals
	Gel	Quick	Mycostatin	
	Oral Suspension	Quick	Nystex	Bristol-Myers Squibb
	Capsule	Controlled	Nilstat	
	Lozenge	Quick	Mycostatin	

Oral mucosal delivery of analgesics has received attention as it is a non-invasive and easy way to treat pain. For example, fentanyl citrate can be applied locally to the oral mucosa to exert rapid analgesia for acute pain. Other drugs delivered via the oral mucosa include sedatives such as midazolam, triazolam and etomidate. They have proven to be clinically effective. Cardiovascular drugs such as nitroglycerin, captopril, verapamil and propafenone have also been studied for potential delivery across the oral mucosa.[5] More recent oral mucosa delivery systems such as aerosols can accurately deliver drugs such as insulin to the mouth. Aerosol formulation

can be quickly absorbed through the buccal mucosal lining and oropharynx regions, providing sufficient plasma insulin levels to control glucose in patients with diabetes.[40]

2.5.2. Vaccine administration via the oral mucosa

Recently, oral mucosa administration of vaccines has been developed to treat infectious diseases. Oral mucosal drug administration can be effective for vaccinations because of its good patient compliance. It is not threatening to children or adults fearful of needles. It is non-invasive and is not prone to needle pricking accidents involving medical staff or the patient. Vaccines administered via the oral mucosa can elicit a systemic immune response in addition to a local immune response at the oral mucosa.[41] Vaccines to treat infectious diseases such as oral candidiasis have also been developed.

2.5.3 Nystatin as an antifungal drug used in the oral mucosa

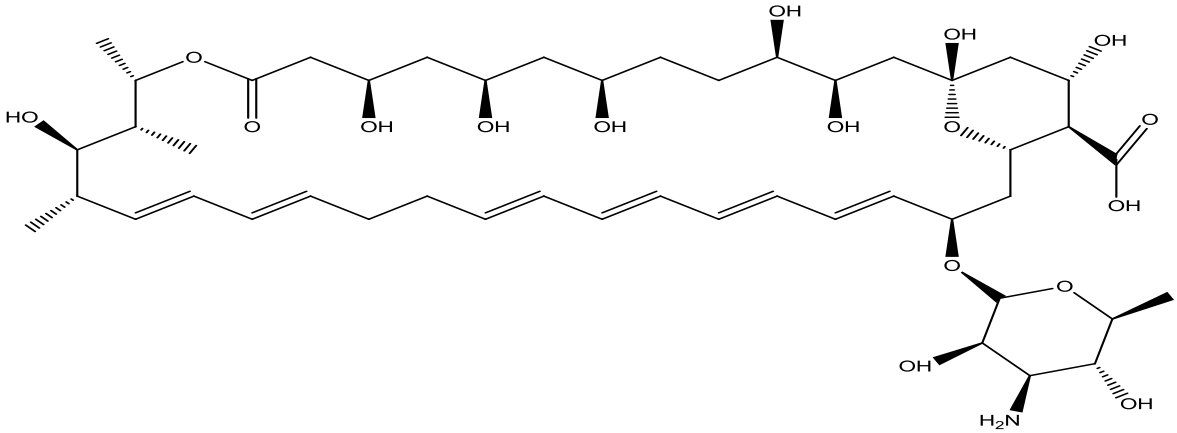


Figure 2.5: Chemical structure of nystatin.

Nystatin is an effective drug used to treat fungal infections such as oral candidiasis (thrush) within the mouth. It consists of a macrocyclic lactone, a hydroxylated tetraene diene backbone, and a mycosamine residue shown in Figure 2.5. Nystatin shows therapeutic activity by binding to the ergosterol component of the fungal cell membrane. As a result, the transmembrane channels expand the intracellular components, leading to fungal cell death.[42] Nystatin is non-

toxic across membranes and has no major side effects beyond potential nausea and vomiting. It comes in a variety of formulations including creams, tablets, oral rinses, gels and pastilles.[43] However, none of these formulations have the capability to interact directly with the oral mucosa, leading to a short residence time and low bioavailability for systemic circulation. Additionally, the current formulations do not provide target specificity for antifungal drug delivery in the oral mucosa, especially in the case of oral rinses. Therefore, the development of a mucoadhesive platform for antifungal delivery may help drug delivery within the oral mucosa.

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

Table 3.1: List of materials

Material	Abbreviation
Polyethylene (glycol) diacrylate (Mn =575 g/mol)	PEGDA
Porcine Type-A Gelatin	Gelatin
1,1,1,3,3,3-Hexafluoro-2-propanol	HFP
Ethanol	Ethanol
De-Ionized Water	DI-Water
Phosphate Buffer Saline	PBS
2,2 dimethoxy-2-phenylacetophenone	DMPA
Dulbecco's Modified Eagle Medium	DMEM
Monopotassium Phosphate	KH_2PO_4
Sodium Chloride	NaCl
Calcium Chloride	$CaCl_2$
Bio-rad Protein Assay Dye Reagent Concentrate	Bio-Rad
Mucin (Gastric)	Mucin
Sodium Hydroxide	NaOH
Nystatin	Nys

3.2 Equipment

Table 3.2: List of equipment

Name	Purpose
MTS Bionix 200 ® Mechanical Testing System	Measure mechanical properties of electrospun scaffolds
Zeiss EVO 50 XVP Scanning Electron Microscope	Obtain high resolution images of nanofibers for morphology characterization and fiber diameter measurement using ImageTool™
Eppendorf Centrifuge Model: 5415D	For Centrifuging materials to be separated into pellet and supernatant phases
UVP Blak-Ray Long Wave UV Lamp 100 W high-pressure mercury vapor filled lamp	UV radiation light source for crosslinking samples
Weighing Balance	Used to measure mass of materials needed
Flexi-Dry FTS System	Freeze dry system to dry frozen samples
Ultra Violet – Visible (UV-Vis) Spectrophotometer	Quantitative tool using light absorption to measure the amount of sample released
10 mL Syringe	To contain gelatin solutions to be pumped during electrospinning
Spellman CZE100R Power Supply	Apply voltage to gelatin solutions for electrospinning
Shaker Plate	To homogenize and mix polymers and solvents
Hot Water Bath	To provide 37°C aqueous environment to simulate drug release in physiological conditions in vitro
12000-14000 MW Dialysis Tubing	For weight specific elution of drug into filtrate

3.3 Experimental methodology

3.3.1 Preparation of gelatin solutions

The electrospinning solutions were prepared first. Briefly, one gram of gelatin was added to 10 mL of HFP. The reaction vial was placed on a shaker plate and shaken continuously for 24 hours to obtain a homogeneous gelatin/HFP solution.[44,45]

3.3.2 Preparation of gelatin-nystatin solutions

One gram of nystatin was added to 10 mL of the gelatin/HFP solution. The mixture solution was vortexed vigorously to completely dissolve nystatin into a homogenous solution. The vial was placed on a shaker plate and shaken continuously for 24 hours.[44,45]

3.3.3 Electrospinning of nanofibers

To fabricate new fibers, the gelatin solution was drawn up through the blunted needle (18 G x 1 ½ in) of a 10 ml syringe. The syringe was loaded into a syringe pump, propelling the gelatin solution out of the needle 125 mm away from the collecting mandrel at a rate of 5 ml/hr. The needle was connected to a positive electrode of a high voltage power supply (Spellman CZE100R, Spellman High Voltage Electronics Corporation). The positive electrode contained a 25 kV voltage that was applied to the needle. This voltage helped the gelatin solution overcome the surface tension at the needle tip. These conditions generated a Taylor cone which allowed a steady stream of gelatin solution to flow from the needle to the grounded collecting plate in a jet-like fashion. As the gelatin solution was being streamed from the needle tip, the HFP solvent evaporated. Randomly aligned nanofibers were collected on a flat, stainless steel mandrel (7.5 cm x 2.5 cm x 0.5 cm (L x W x T)) rotating at ~500 rpm. (Figure 3.1)

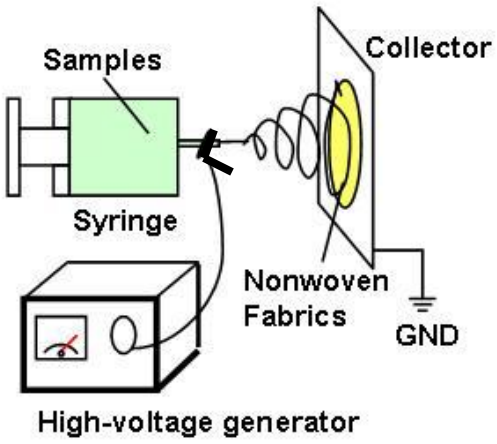


Figure 3.1: Electrospinning apparatus setup.

Once electrospinning was complete, the gelatin scaffolds were carefully removed from the steel mandrel using a razor blade and stored under the degassing shelf.

3.3.4 Crosslinking of scaffolds

Gelatin is a hydrophilic polymer vulnerable to dissolution in aqueous solutions. Crosslinking gelatin is necessary to maintain its mechanical stability. In this study, 1X, 2X, 4X, 8X crosslinking solutions were made by varying amounts of PEG-diacrylate (MW = 575 g/mol) and DMPA photoinitiator in 2 ml of ethanol (Table 3.3). The crosslinking solution was poured on a rectangular nanofiber scaffold (7.5 x 2.5 cm) and allowed to incubate for 30 min. Next, the scaffold from a 14 cm distance was held under UV light (UVP Blak-Ray Long Wave Lamp, 100 Watts) for 2 minutes on each side of the scaffold. DMPA catalyzed the reaction through its decomposition after exposure to UV light. The methylated DMPA segment activated the acrylate double bond of the PEG-DA monomer to initiate crosslinking polymerization. The propogation of PEG-DA monomers eventually formed a stable network with the gelatin embedded. This crosslinking mechanism is shown in Figure 3.2. It involves four steps: decomposition, initiation, propagation and termination.

Table 3.3: Crosslinker amounts per 2 ml of ethanol.

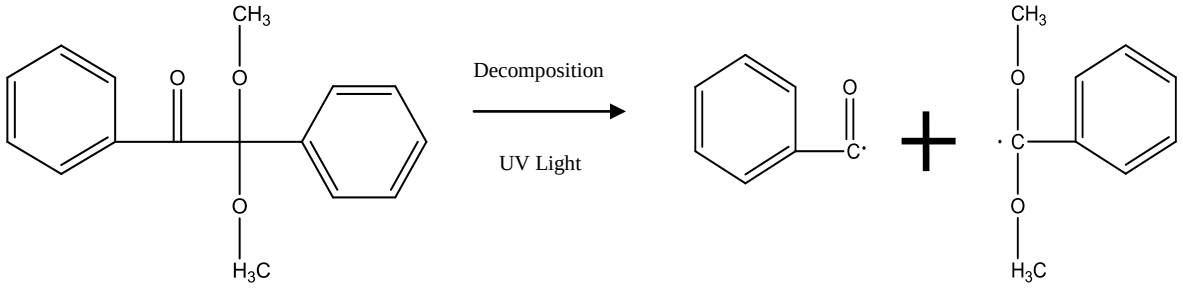
Crosslinking Amounts	1X Concentration	2X Concentration	4X Concentration	8X Concentration
PEG Diacrylate Mass	0.056g/ml	0.112g/ml	0.224g/ml	0.448g/ml
DMPA Mass	2 mg/ml	4 mg/ml	8 mg/ml	16 mg/ml
PEG Diacrylate Concentration	5% (v/v)	10% (v/v)	20% (v/v)	40% (v/v)
DMPA Concentration	0.2% (w/v)	0.4% (w/v)	0.8% (w/v)	1.6% (w/v)

*all 7.5 x 2.5 cm scaffolds were incubated for 30 minutes before exposure to UV light

Table 3.4: Different incubation times.

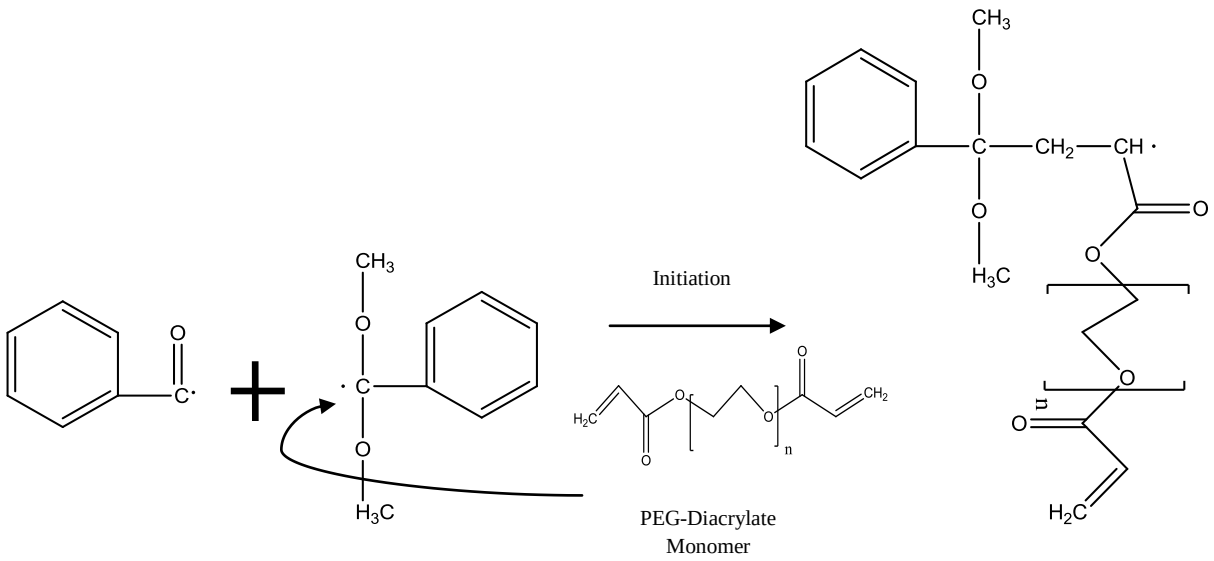
Incubation Time	30 minutes	12 hours	24 hours
	30 minutes between adding 1X crosslinker solution on scaffold and exposure to UV light	12 hours between adding 1X crosslinker solution on scaffold and exposure to UV light	24 hours between adding 1X crosslinker solution on scaffold and exposure to UV light

*all 7.5 x 2.5 cm scaffolds were crosslinked with 2 mL 1X concentration of PEG diacrylate and DMPA

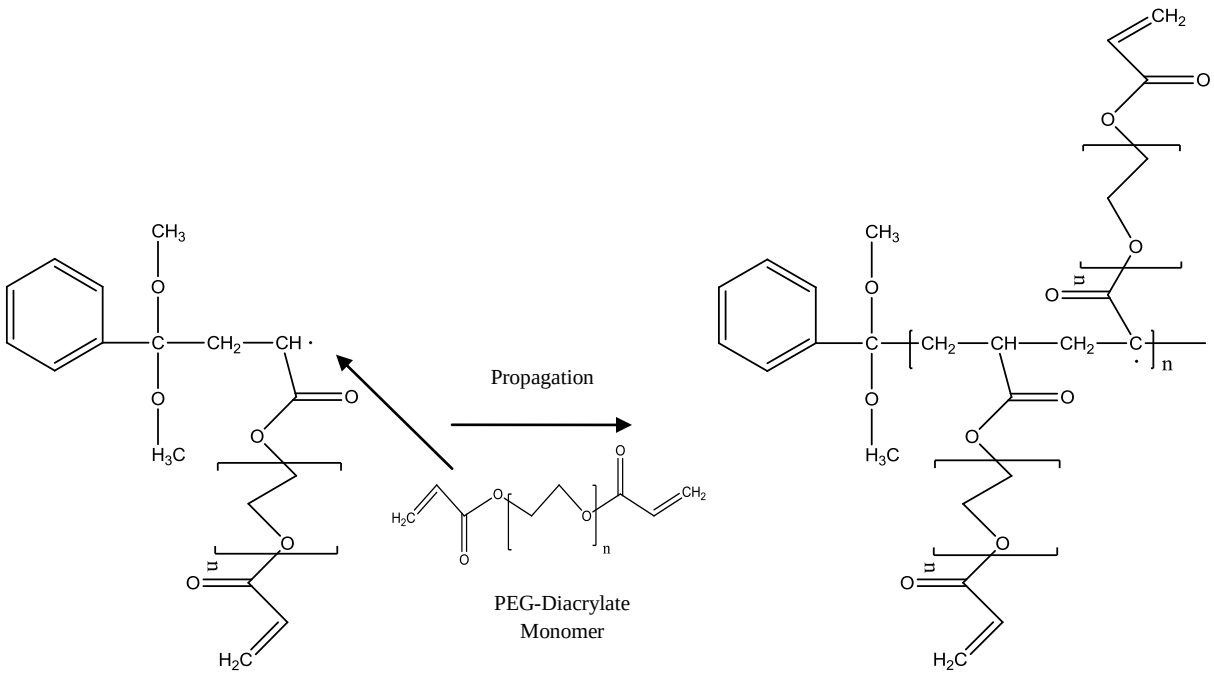


DMPA

Step 1


Initiation
PEG-Diacrylate
Monomer

Step 2


Propagation
PEG-Diacrylate
Monomer

Step 3

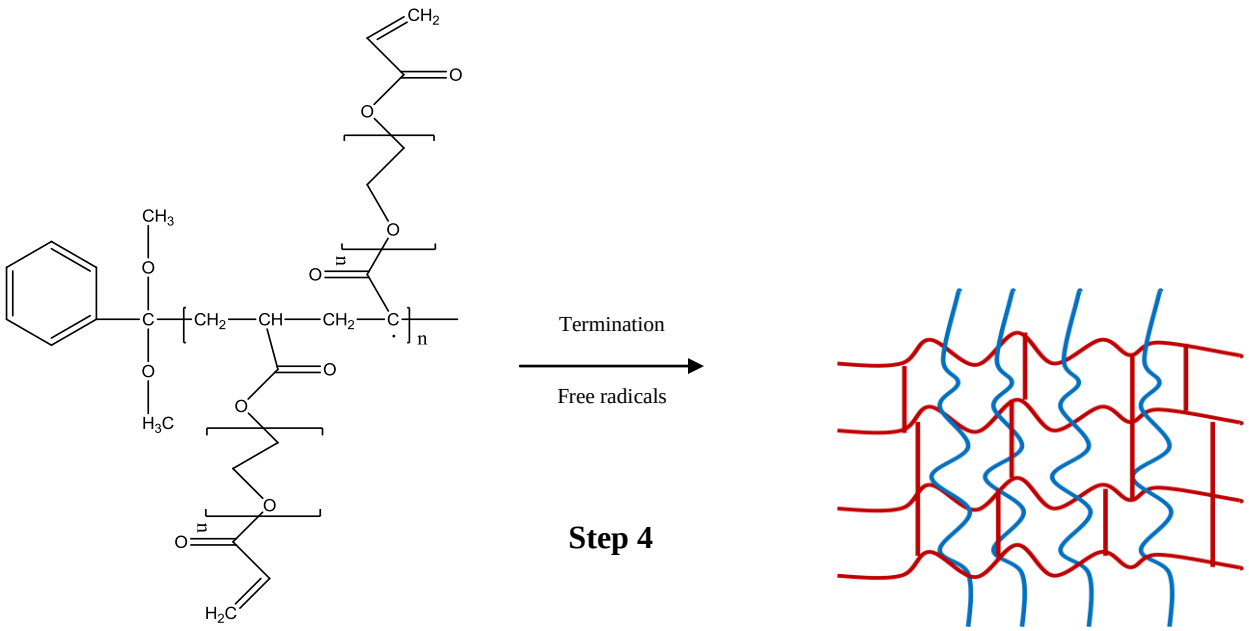


Figure 3.2: Crosslinking mechanism of PEG-diacrylate. (Modified from [46])

3.4 Characterization

3.4.1 SEM

Prior to SEM imaging, nanofiber scaffolds were placed on a 1 cm diameter stub. The stub was placed on a specimen holder and gold sputter coated. SEM images were taken on the Zeiss EVO 50 XVP Scanning Electron Microscope**.** Once the SEM images were compiled, UTHSCSA ImageTool$^{TM\ software}$ was used to measure 60 randomly chosen fibers to determine each diameter of fibers within the scaffold.

3.4.2 Tensile testing

The mechanical properties of nanofiber scaffolds were tested using the MTS Bionix 200 ® Mechanical Testing System in conjunction with TestWorks 4.0 software. The nanofiber scaffolds (n = 10) were cut into 20 mm length dog-bone shapes using a punch die

(ODC Testing & Molds) with dimensions .75, .125 and .24 inches at its length, narrowest point and widest point respectively. The scaffolds mechanical properties including peak load, peak stress, modulus, strain at break and energy to break were obtained.

3.4.3. In vitro degradation studies

The in vitro degradation of scaffolds was evaluated three different media: i) Dulbecco's modified Eagle's medium (DMEM), (DMEM) supplemented with 10% fetal bovine serum (FBS) and simulated saliva fluid (SSF) consisting of KH2PO4, NaCl, CaCl2 and NaOH ions at 37° C.[47] Samples of 1 cm diameter (n=9) from each crosslinked nanofiber scaffold were weighed out and then individually immersed in a well filled with 1.5 mL of one of the three solutions. At 6 hr, 12 hr and 24 hr time-points, samples were taken out and centrifuged for 20 minutes. After centrifugation, they were frozen, lyophilized, and weighed. The amount of weight loss due to degradation was calculated according to the following formula:

$$\text{Weight loss due to degradation (\%)} = [(W_o - W_d) / W_o] \times 100 \quad (2)$$

Where W_o = original weight of the sample, and W_d = weight of the sample after degradation

3.4.4 Porosity studies

Gelatin nanofiber (1 cm x 1 cm) samples (n=5) were cut out and weighed. Their thickness was measured with a digital caliper. The apparent volume (Va) of the scaffold was then determined. The volume of the material was determined based on collagen's density of 1.41 g/cm^3.[48] The porosity in terms of apparent void volume fraction is given below.

$$\text{Porosity} = [1 - (V_g / V_a)] \times 100 \quad (3)$$

Where V_g = mass/material's density, and V_a= 1 cm x 1 cm x thickness

3.4.5 Swelling studies

Nanofiber (1 cm x 1 cm) samples (n=5) were placed in wells filled with 5 mL of pH 7.4 PBS (one sample per well) at room temperature. They were taken out and immediately blot dried at predetermined time points up to 24 hours, weighed, and placed back in the solution. Swelling ratio is determined as the ratio of mass of swollen sample at a given time point to the mass of the dry sample.

3.4.6 Mucoadhesion studies

Mucoadhesion of the scaffold was tested by examining attachment of mucin proteins to the scaffold. Mucin solution with concentration 1 mg/mL was prepared by weighing out 4 mg of mucin dissolved in 1 mL of de-ionized water. The 4 mg/mL solution was then diluted to the 1 mg/mL concentration by taking a 250 µL aliquot from the 4 mg/mL solution and adding it to 200 µL of protein assay dye reagent (Bio-rad) and 800 µL of de-ionized water in a 2 mL eppendorf tube. The reaction vessel was then vortexed to mix the mucin, DI water and dye reagent components. 4X and 8X crosslinked scaffolds (n=8) were immersed in 1 mg/mL mucin solution and taken out immediately. The mucin concentration in the remaining solution was assessed by Bio-rad assay under the UV-Vis spectrophotometer. PEG only hydrogels, (1 cm diameter, (n=8)) were used as a negative control since PEG confers a non-fouling, protein resistant surface. These hydrogels were composed of 7.5% (w/v) PEG-1500 and 2% (w/v) DMPA photoinitiator in 2 mL of solution containing 1 mL of PEG-DA and water each. In addition, 4X crosslinked gelatin hydrogels were used for comparison to evaluate if surface architecture influences mucoadhesion. The amount of mucin absorbed onto scaffold was then indirectly determined by the mucin concentration change before and after sample immersion.

3.4.7 Drug release studies

The 4X nanofiber crosslinked scaffolds were chosen for this study because of its good stability in aqueous solutions. These scaffolds containing different amounts of nystatin were immersed in PBS (pH 7.4) and loaded into dialysis tubing with molecular weight cutoff at (MWCO) 12000-14000 Da. The dosage amounts were varied around the prescribed 100 mg Nystatin dosage recommended by the U.S. National Library of Medicine.[49] These dosages were varied to analyze and compare its influence on the drug release profiles, particularly within the first six hours. At predetermined time points, a 1 mL of aliquot was withdrawn from the release medium for UV-Vis measurement. One mL of fresh PBS, pre-equilibrated at 37°C was immediately added to maintain its volume. Absorbance of nystatin was measured at 305 nm as reported in literature.[42] The absorbance of the withdrawn solution was measured at each time-point and referenced against the standard curve to indicate the cumulative amount (mg) of drug release shown by the following equation:

$$cumulative\ \%\ release = \frac{0.2L \times [concentration]_{cumulative} + 0.01L \times [concentration]_{aliquot}}{Initial\ \ amount\ of\ \ Nys\ in\ sample} \qquad (4)$$

The loading amount of nystatin was determined as follows. First, a 10 mm diameter scaffold was immersed in 10 mL of PBS for over 24 hours to allow complete release of the drug. Next, 100 µL of the dissolved nystatin solution was extracted and added with 900 µL of PBS (pH 7.4) to dilute the sample for measurement preparation. Lastly, the sample's nystatin absorbance was measured then referenced against the standard curve (Appendix A.3) to find the corresponding concentration. The absorbance and concentration was found to be 1.316 and .235 mg/mL respectively .The concentration was multiplied by a factor of 100 to account for

extracting 1 mL of sample from 10 mL of solution and diluting it by another factor of 10. The total mass of nystatin eluted from a 10 mm diameter (78.54 mm^2 area) nanofiber scaffold in 10 mL of solution was 23.5 mg. The initial amounts of nystatin were set at 50, 100, 200 and 400 mg dosages through creating drug loaded nanofiber scaffolds with corresponding areas (167 mm^2, 334 mm^2, 668 mm^2, 1336 $mm^{2)}$.

3.4.8 Statistical analysis

Statistical analysis was evaluated on mean scaffold fiber diameters, tensile property values, degradation, porosity, swelling mucoadhesion and drug release kinetics. All statistical analysis was done using one-factor analysis of variance (ANOVA) and Tukey's test for significance using SigmaPlot 12. P-values less than 0.05 were considered statistically significant. Graphical representations of mean data were constructed with Microsoft Excel 2007 with error-bars representing standard deviations. Statistical analysis data is provided in Appendix B.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Effects of incubation time of crosslinker

4.1.1 Effect on morphology

In the crosslinking step, the concentration of PEG-DA and DMPA photoinitiator was kept constant at 1X while incubation time of nanofiber scaffolds with crosslinking solution was varied. The morphology of the noncrosslinked and crosslinked gelatin nanofiber scaffolds was examined by SEM. The untreated scaffolds had the greatest fiber diameter. Increasing incubation time had a negligible influence on fiber morphology. No discernible differences in fiber morphology among crosslinked and non-crosslinked nanofibers were observed. Incubation time increase did not affect the nanofiber definition or its porous network.

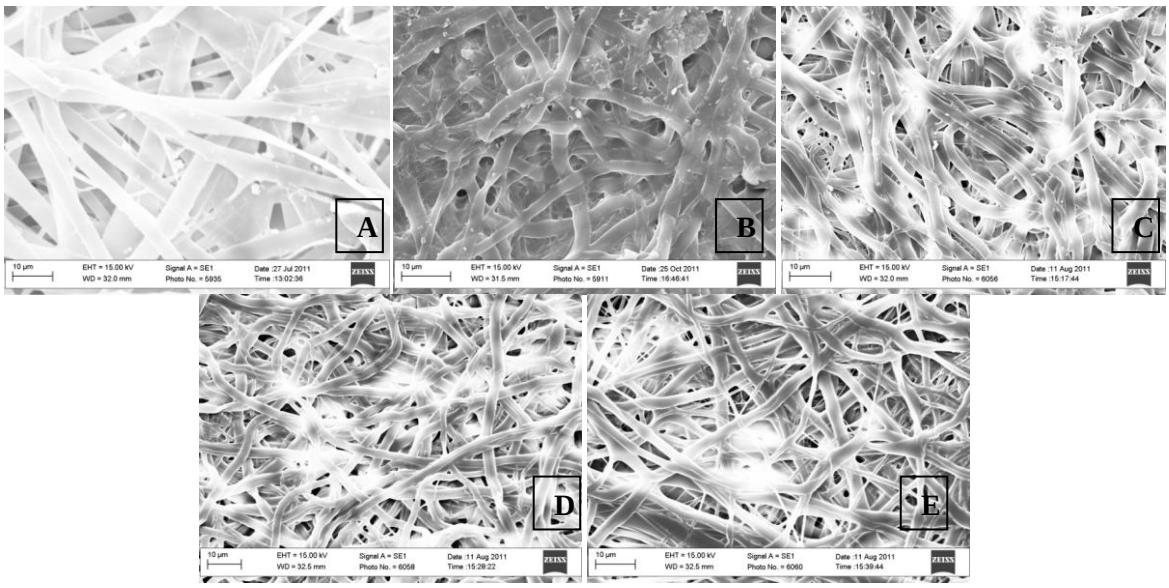


Figure 4.1: SEM image of (A) electrospun gelatin nanofibers with no crosslinking treatment, (B) electrospun gelatin nanofiber mats crosslinked with ethanol, (C) electrospun gelatin nanofiber mats crosslinked with 1X concentration of PEG-DA & DMPA before 30 minute incubation, (D) before 12 hour incubation, (E) before 24 hour incubation. Scaffolds (B-E) after incubation were exposed to UV light treatment for two minutes on each side. Bars: 10µm.

Structural stability of crosslinked gelatin nanofiber scaffolds in three types of media was also investigated. A set of SEM images of scaffolds having been immersed in DMEM for 24 hours are presented here. (Figure 4.2) Non-crosslinked and 30 minute-incubated scaffolds were not shown due to their complete degradation. The pores are uniformly distributed across the matrix. However, these scaffolds lost a nanofiber network and became more porous.

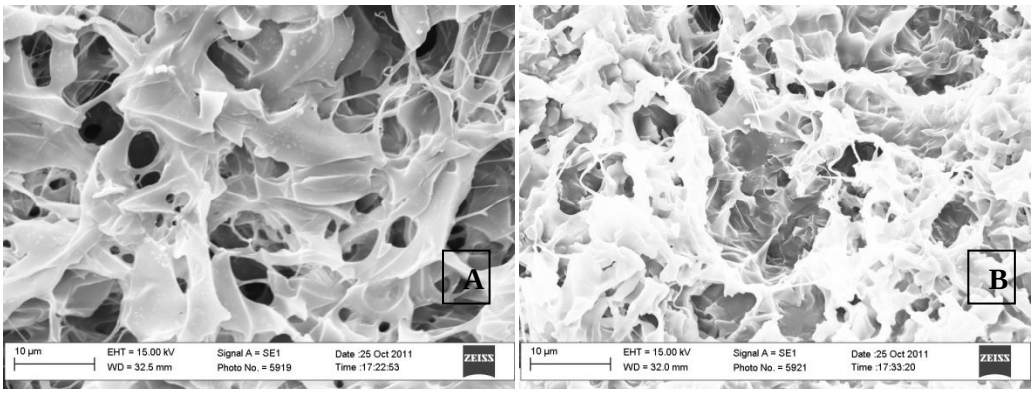


Figure 4.2: SEM images of (A) degraded 12 hour incubation treated crosslinked electrospun gelatin nanofiber mats after 24 hours immersion in DMEM, (B) degraded 24 hour incubation treated crosslinked electrospun gelatin nanofiber mats after 24 hours immersion in DMEM All scaffolds (A, B) after incubation were exposed to UV light treatment for two minutes on each side. Bars: 10µm.

4.1.2 Effect on fiber diameter

Ethanol was used to dissolve crosslinking reagents. Its impact on crosslinked scaffolds was studied as well. Incubating uncrosslinked nanofibers in ethanol alone for 30 minutes resulted in a decrease in fiber diameter. This was possibly due to nanofiber shrinkage in ethanol. The network still maintained a porous structure and fiber morphology. Increasing incubation time to 24 h in ethanol in the presence of crosslinking reagents further decreased the fiber diameter. There was a 28% decrease in fiber diameter when incubation time was increased from 12 to 24 hours. Significant statistical differences ($p<0.05$) were shown for uncrosslinked scaffolds tested vs. all groups except ethanol. These differences were also shown for 24 hour incubated scaffolds tested vs. all groups.

Sample	Uncrosslinked	Ethanol (30 min)	30 minute incubation	12 hour incubation	24 hour incubation
Mean (μm)	3.22 ± 1.53	2.45 ± 0.720	2.27 ± 0.790	2.37 ± 0.750	1.70 ± 0.790

Table 4.1: Fiber diameter as a function of incubation time.

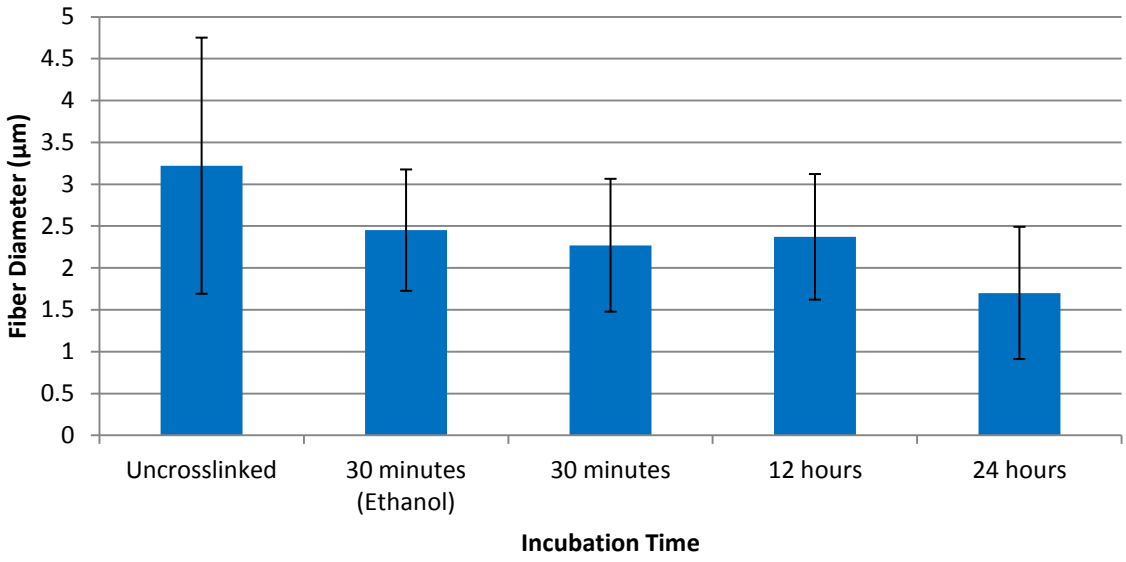


Figure 4.3: Fiber diameter measurements recorded from uncrosslinked and crosslinked electrospun gelatin nanofiber mats of varying incubation times with two minutes of UV radiation on each side. All crosslinked scaffolds except ethanol control were treated with 1X concentration of PEG-DA & DMPA**.**

4.1.3 Effect on tensile properties

The data acquired from mechanical testing confirmed ethanol and incubation time of crosslinking reagents significantly changed mechanical properties compared to the untreated gelatin nanofiber scaffolds. Specifically, increasing the incubation time had an inverse effect on the peak stress and modulus. As shown in Table 4.2, the peak stress increased from 1.759 to 18.36 MPa, showing a ten-fold increase by ethanol alone. The modulus also increased from to 606.5 MPa. However, peak stress and modulus declined as incubation time was increased particularly with crosslinking reagents. Peak stress was 17.48 MPa for incubated samples then

decreased to 17.23 and 11.29 MPa at 12 and 24 hour incubated samples respectively. The 30 minute incubated samples had a modulus of 554.5 MPa. Increasing the incubation time to 12 hours slightly decreased the modulus to 523.1 MPa while samples incubated for 24 hours had a significant drop in modulus to 367.0 MPa. The drop in peak stress and modulus after increasing incubation time can be attributed to the hardening effect of PEG-DA, ethanol and photoinitiator crosslinking agents on the scaffold. Strain at break shown in Figure 4.5 showed there was an increase by 0.0174 mm/mm when ethanol was used compared to uncrosslinked scaffolds. For the 30 minute incubation treated scaffolds, their strain at break increased further to 0.0460 mm/mm. There was no appreciable change in strain at break when the incubation time was extended from 30 minutes to 12 and 24 hours. A noticeable trend in the scaffold's peak stress, modulus and strain at break did develop when incubation time of crosslinking reagents was increased, while the concentration of crosslinking solution was kept constant. After peak stress and modulus testing, statistical differences ($p<0.05$) were shown for uncrosslinked scaffolds evaluated against all subgroups except 24 hour incubated samples. The majority of subgroups evaluated for peak load shown no significant statistical differences when tested against each other.

Table 4.2: Tensile properties as a function of incubation time.

Sample	Thickness (in)	Peak Load (N)	Peak Stress (MPa)	Modulus (MPa)	Strain At Break (mm/mm)	Energy to Break (N*mm)
Untreated	0.00915 ± 0.00120	1.062 ± 0.5374	1.759 ± 1.004	94.28 ± 41.41	0.0286 ± 0.0120	0.1045 ± 0.07620
Ethanol	0.0130 ± 0.00670	16.04± 6.791	18.36± 9.266	606.5± 201.9	0.0460± 0.0134	3.199±1.989
30 minute incubation	0.0200 ± 0.00270	22.07 ± 5.416	17.48 ± 4.758	554.5 ± 153.8	0.0580 ± 0.0180	5.758 ± 1.842
12 hour incubation	0.0140 ± 0.00180	15.82 ± 3.975	17.23 ± 0.8915	523.1 ± 153.6	0.0590 ± 0.0200	4.350 ± 2.127
24 hour incubation	0.0280 ± 0.0114	18.80 ± 6.006	11.29 ± 4.820	367.0 ± 203.7	0.056 ± 0.0181	4.633 ± 2.263

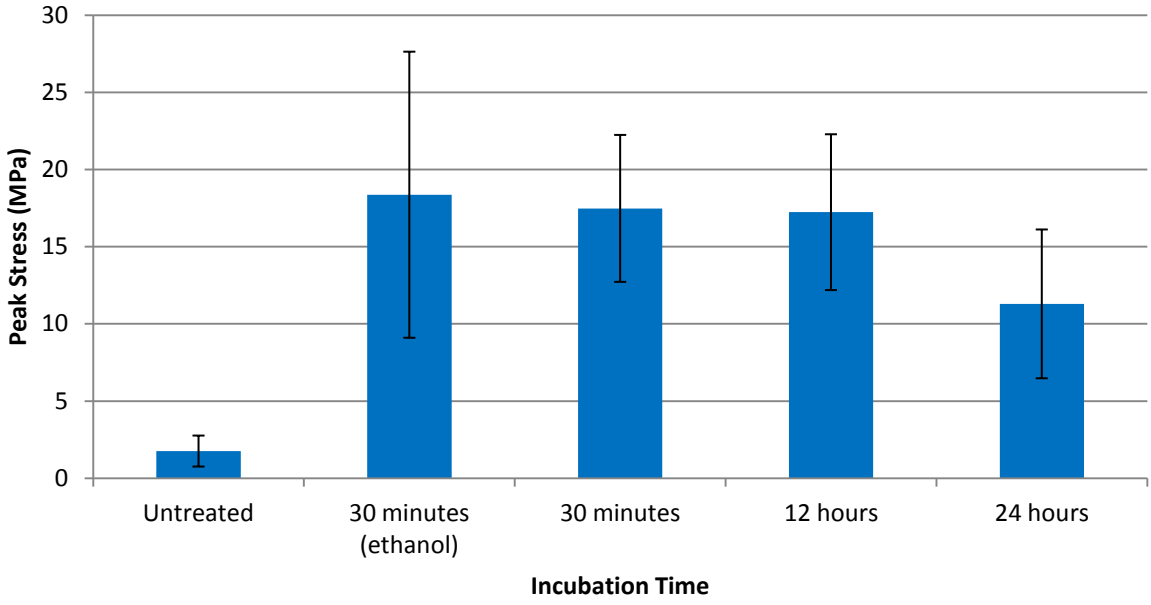


Figure 4.4: Peak stress measurements recorded from uncrosslinked and crosslinked electrospun gelatin nanofiber mats of varying incubation times with two minutes of UV radiation on each side. All crosslinked scaffolds except ethanol control were treated with 1X concentration of PEG-DA & DMPA**.** All treated samples dried after crosslinking treatment.

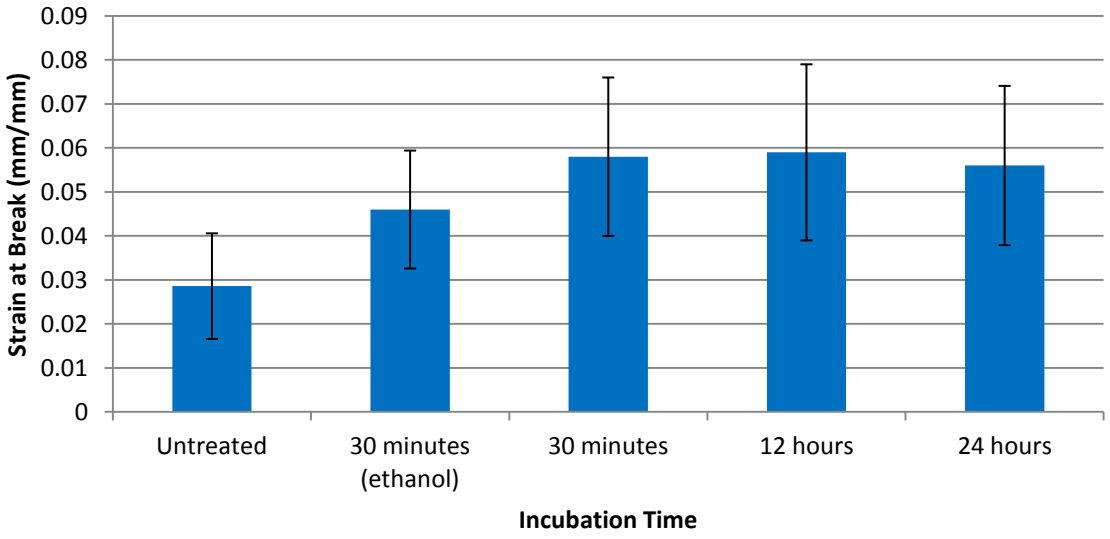


Figure 4.5: Strain at break measurements recorded from uncrosslinked and crosslinked electrospun gelatin nanofiber mats of varying incubation times with two minutes of UV radiation on each side. All crosslinked scaffolds except ethanol control were treated with 1X concentration of PEG-DA & DMPA**.** All treated samples dried after crosslinking treatment.

4.1.4 Effect on in vitro degradation

In vitro degradation studies were conducted to evaluate the scaffold's stability in several media conditions at 37°C. Uncrosslinked control and 30 minute incubated samples completely degraded in six hours. A longer incubation of crosslinking reagents slowed down the degradation progress due to a more stabilized crosslinked structure. The matrix allowed water to diffuse through the scaffold, breaking up the water soluble gelatin nanofiber network at 37° C. This observation was consistent with the swelling data shown in Figure 4.10. The 30 minute-incubated samples, the control and ethanol uncrosslinked samples exhibited a much higher degree of swelling than the 12 hour and 24 hour-incubated samples. Significant statistical differences ($p<0.05$) were shown for 30 minute incubated samples tested against other subgroups evaluated in all media.

Table 4.3: In vitro degradation in DMEM + 10% FBS as a function of incubation time.

Immersion Time in Media	30 minute incubation	12 hour incubation	24 hour incubation
6 hours	100% ± 0.00	77.10% ± 1.760%	78.29% ± 7.440%
12 hours	100% ± 0.00	78.22% ± 8.510%	81.05% ± 7.480%
24 hours	100% ± 0.00	81.94% ± 3.490%	81.65% ± 5.880%

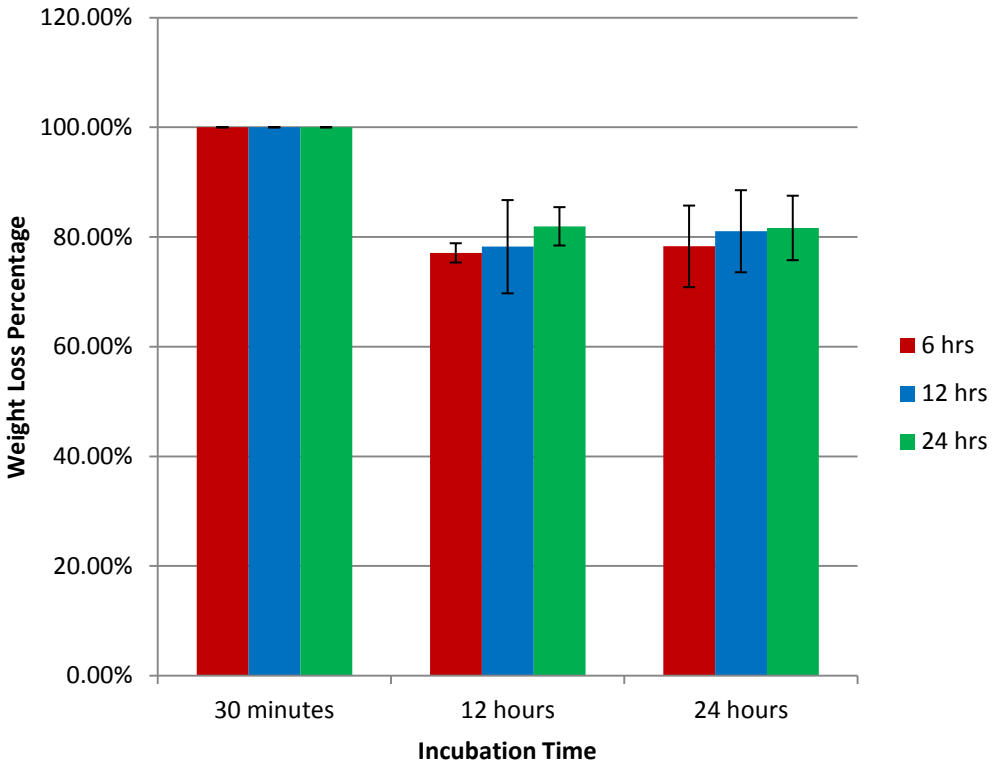


Figure 4.6: Degradation rate of crosslinked electrospun gelatin nanofiber mats of varying incubation times with two minutes of UV radiation on each side. All crosslinked scaffolds were treated with 1X concentration of PEG-DA & DMPA**.** Scaffold degradation followed after immersion into DMEM + 10% fetal bovine serum. Scaffold degradation followed after immersion into DMEM. Degradation is calculated as $D(\%) = 100 \times (w_o - w_d)/w_o$, where w_o and w_d are the weight of the nanofibers before and after degradation at selected time points.

Table 4.4: In vitro degradation in simulated salivary fluid as a function of incubation time.

Immersion time in media	30 minute incubation	12 hour incubation	24 hour incubation
6 hours	100% ± 0.00	83.48% ± 3.030%	83.25% ± 5.600%
12 hours	100% ± 0.00	84.32% ± 6.940%	85.60% ± 6.380%
24 hours	100% ± 0.00	84.22% ± 3.130%	83.53% ± 0.300%

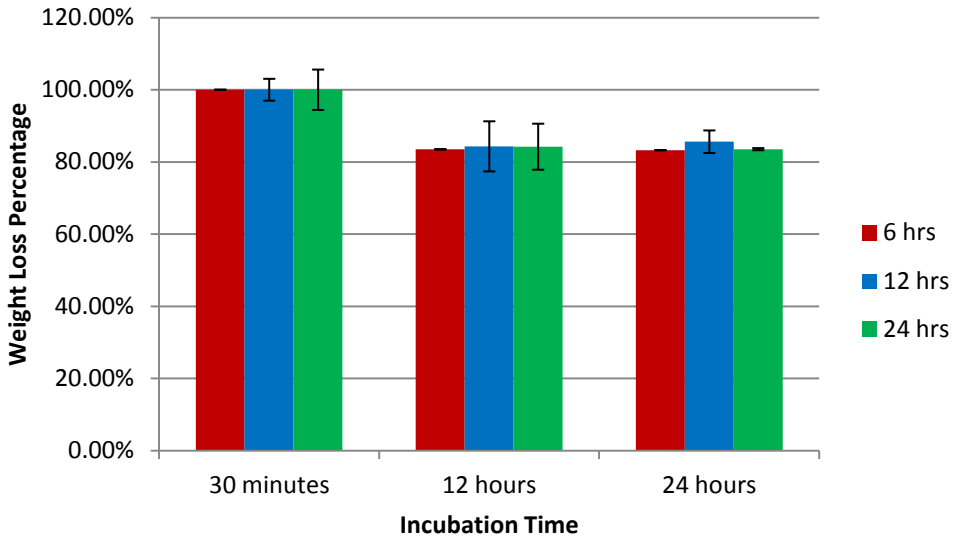


Figure 4.7: Degradation rate of crosslinked electrospun gelatin nanofiber mats of varying incubation times with two minutes of UV radiation on each side. All crosslinked scaffolds were treated with 1X concentration of PEG-DA & DMPA**.** Scaffold degradation followed after immersion into simulated salivary fluid. Scaffold degradation followed after immersion into DMEM. Degradation is calculated as $D(\%) = 100 \times (w_o - w_d)/w_o$, where w_o and w_e are the weight of the nanofibers before and after degradation at selected time points.

Table 4.5: In vitro degradation in DMEM control as a function of incubation time.

Immersion time in media	30 minute incubation	12 hour incubation	24 hour incubation
6 hours	93.69% ± 5.720%	83.48% ± 5.720%	83.25% ± 1.610%
12 hours	83.40% ± 1.450%	84.32% ± 1.200%	85.60% ± 2.430%
24 hours	96.80% ± 5.540%	84.22% ± 1.380%	83.53% ± 3.430%

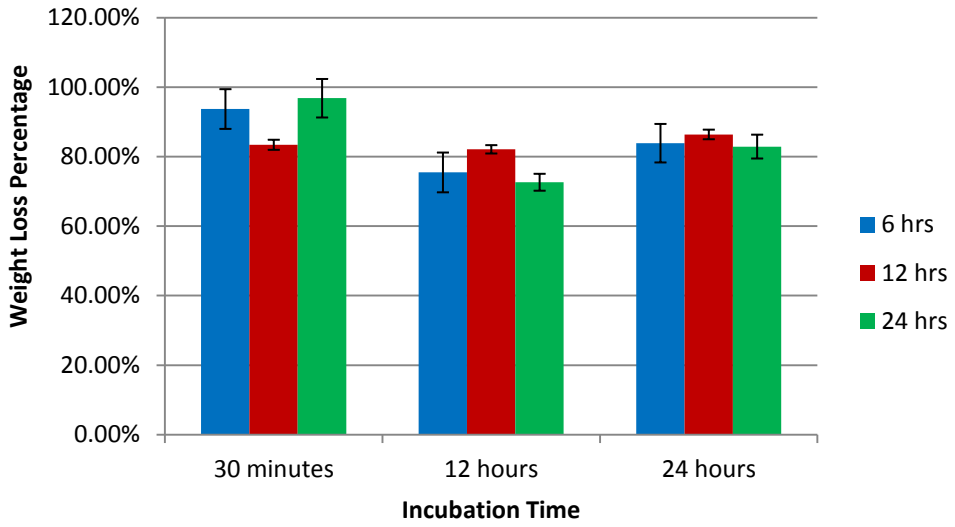


Figure 4.8: Degradation rate of crosslinked electrospun gelatin nanofiber mats of varying incubation times with two minutes of UV radiation on each side. All crosslinked scaffolds were treated with 1X concentration of PEG-DA & DMPA**.** Scaffold degradation followed after immersion into DMEM. Degradation is calculated as: $D(\%) = 100 \times (w_o - w_d)/w_o$, where w_o and w_e are the weight of the nanofibers before and after degradation at selected time points.

4.1.5 Effect on porosity

Untreated and ethanol treated samples incubated for 30 minutes had an average porosity of 82.15 and 84.58%, respectively. The average porosity of scaffolds incubated with crosslinking reagents for 12 hours was 37.12 %. However, 24 hour-incubated samples showed a higher porosity than the 12 hour samples, presumably due to a possible degradation of the scaffold in ethanol. All subgroups tested showed significant statistical differences ($p<0.05$) with exception to 30 minute incubated samples vs. 24 hour incubated samples and ethanol vs. uncrosslinked samples.

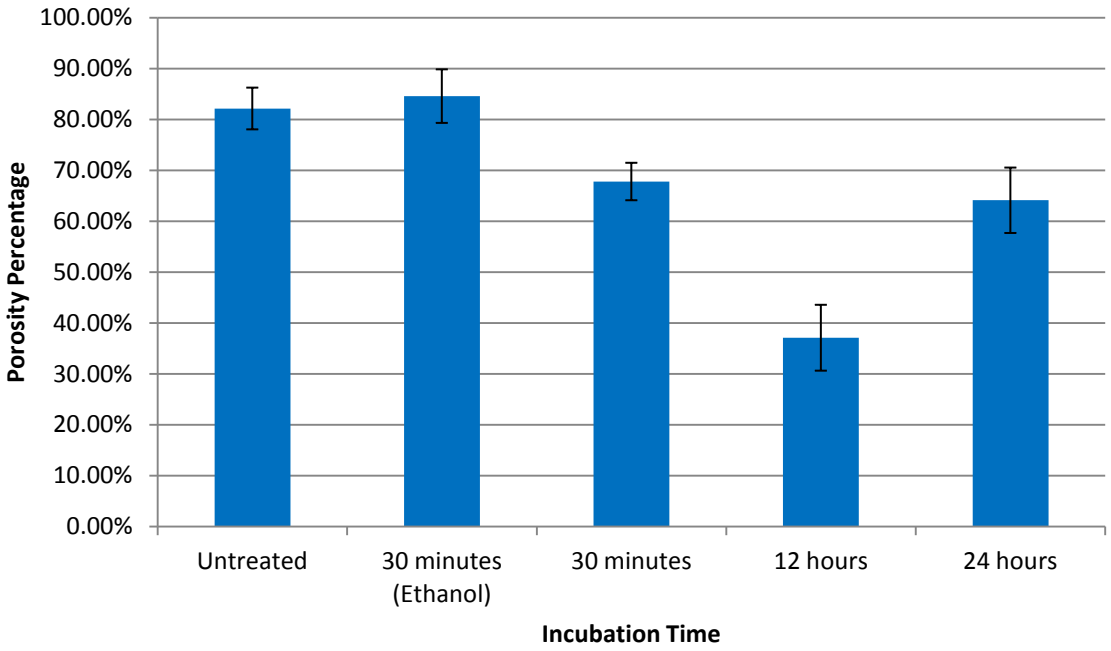


Figure 4.9: Porosity measurements recorded from uncrosslinked and crosslinked electrospun gelatin nanofiber mats of varying incubation times with two minutes of UV radiation on each side. All crosslinked scaffolds except ethanol control were treated with 1X concentration of PEG-DA & DMPA**.**

4.1.6 Effect on swelling

Scaffolds incubated for 30 minutes had higher swelling rates than scaffolds incubated for 12 and 24 hours. However, there was no significant difference in swelling when the incubation time was increased from 12 to 24 hours. After 24 hours in PBS, all samples with exception to untreated samples sustained a constant absorption profile as the graph reached a plateau after roughly 2 hours in PBS. Untreated samples had the highest swelling ratio among all samples examined. Significant statistical differences ($p<0.05$) were shown for uncrosslinked treated scaffolds tested against all other subgroups.

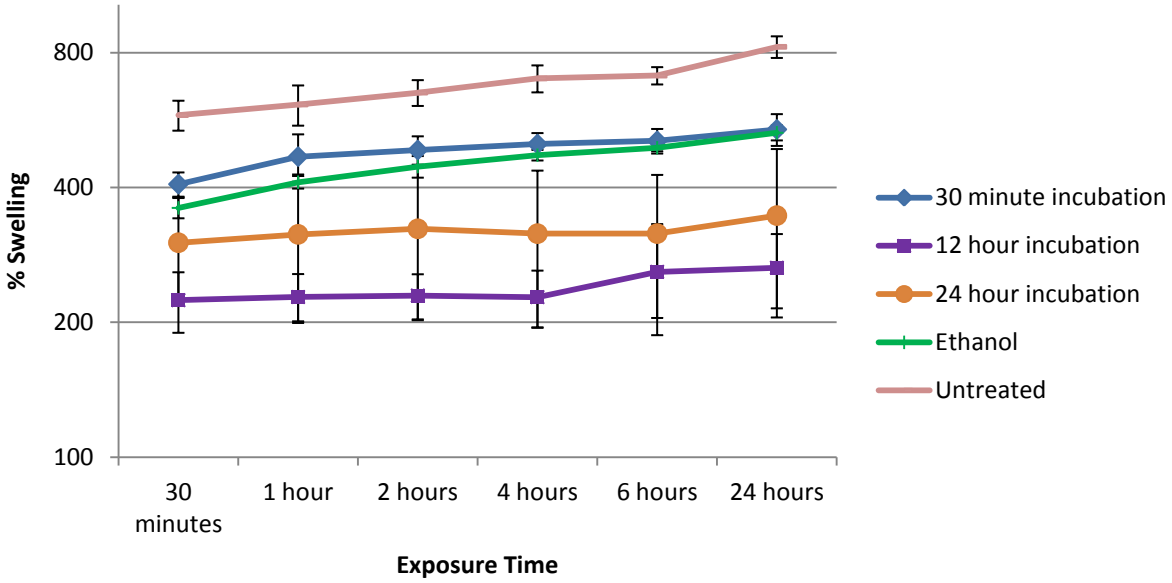


Figure 4.10: Swelling kinetics of crosslinked electrospun gelatin nanofiber mats of varying incubation times with two minutes of UV radiation on each side. All crosslinked scaffolds were treated with 1X concentration of PEG-DA & DMPA**.** Scaffold swelling followed after immersion into PBS at room temperature. Swelling is calculated as $S(\%) = 100 \times (m_o - m_e)/m_o$, where m_o and m_e are the weights of the dry and swollen nanofibers respectively at selected time points.

4.2 Effects of crosslinker concentration

4.2.1 Effect on morphology

The 1X treatment caused the scaffolds to lose its definition due to the fusion of fibers within the network caused by the crosslinking solution. However, the nanofiber scaffold's morphology did not fully collapse and its structure was conserved during crosslinking. The crosslinked nanofiber scaffolds at the 8X concentration did not deviate much in morphology compared to corresponding scaffolds at lower crosslinker concentrations. Fusion of fibers confirmed the crosslinking between gelatin, PEG-DA and DMPA without the morphology being compromised.

Morphology degradation of crosslinked scaffolds in different media was also studied. Regardless of crosslinker concentration and medium, gelatin nanofibers completely fused into an

undefined mesh after being immersed. The 1X scaffold completely degraded within six hours. There were no available samples from that formulation to image under SEM. Figure 4.12 confirmed the loss of the scaffold's nanofiber network. One possible postulation is hydrophilic gelatin interacts directly with the surrounding solution, allowing water molecules to penetrate the porous gelatin network and causing fiber expansion and swelling within the scaffold. The enzymes within the cell culture media were another factor to accelerate degradation of gelatin nanofibers.

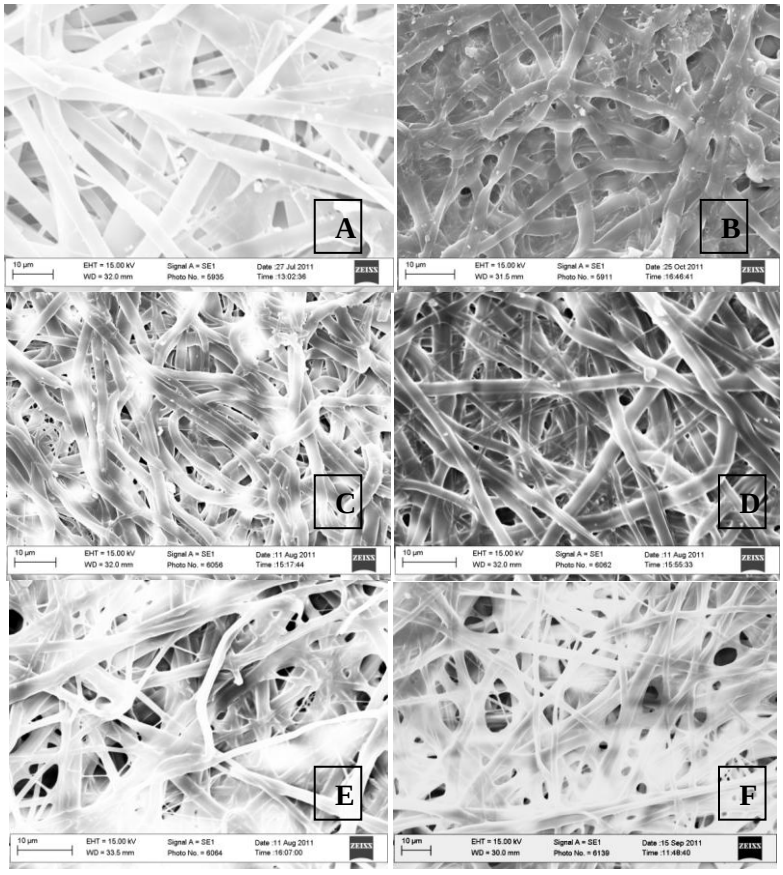


Figure 4.11: SEM image of (A) electrospun gelatin nanofibers with no crosslinking treatment, (B) electrospun gelatin nanofiber mats crosslinked with ethanol (C) 1X concentration of PEG-DA & DMPA, (D) 2X concentration of PEG-DA & DMPA, (E) 4X concentration of PEG-DA & DMPA, (F) 8X concentration of PEG-DA & DMPA Scaffolds (B-F) after crosslinking reagent exposure were then incubated for 30 minutes before UV light treatment for two minutes on each side. Bars: 10µm.

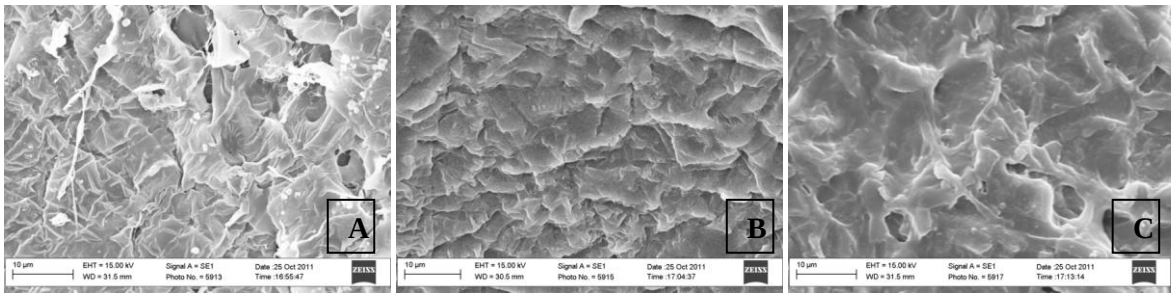


Figure 4.12: After 24 hours immersion in DMEM, SEM images of (A) degraded electrospun gelatin nanofiber mats crosslinked with 2X concentration of PEG-DA & DMPA, (B) degraded electrospun gelatin nanofiber mats crosslinked with 4X concentration of PEG-DA & DMPA, (C) degraded electrospun gelatin nanofiber mats crosslinked with 8X concentration of PEG-DA & DMPA**.** All scaffolds (A-C) after crosslinking reagent exposure were then incubated for 30 minutes before UV light treatment for two minutes on each side. Untreated and 1X scaffolds not shown due to complete degradation. Bars: 10µm.

4.2.2 Effect of fiber diameter

The morphology of crosslinked modulated electrospun gelatin nanofiber scaffolds was further scrutinized through fiber diameter examination. Fiber diameter decreased when gelatin nanofibers were incubated in ethanol alone or with crosslinker. There was a 24% decrease in diameter of ethanol treated scaffolds as compared to uncrosslinked nanofibers. When 1X crosslinker was added to ethanol and used to crosslink nanofibers, the mean fiber diameter decreased to 2.27 µm. Shown in Table 4.6 and Figure 4.13, 4X crosslinker concentration resulted in the lowest fiber diameter. All uncrosslinked and 1X concentrated scaffolds were significantly different ($p<0.05$) when evaluated against other subgroups with the exception of ethanol.

When crosslinker concentration was increased from 1X to 8X, the fiber diameter slightly decreased but the morphology of the crosslinked nanofiber scaffolds was retained. No significant morphological differences were observed across crosslinked scaffolds. (Figure 4.11) The scaffolds from each individual treatment displayed fiber fusion, confirming crosslinking within the network and a well distributed porous structure.

Table 4.6: Fiber diameter as a function of crosslinker concentration.

Sample	Uncrosslinked	Ethanol	1X	2X	4X	8X
Mean (µm)	3.22 ± 1.53	2.45 ± 0.720	2.27 ± 0.790	1.69 ± 0.900	1.46 ± 0.790	2.26 ± 1.19

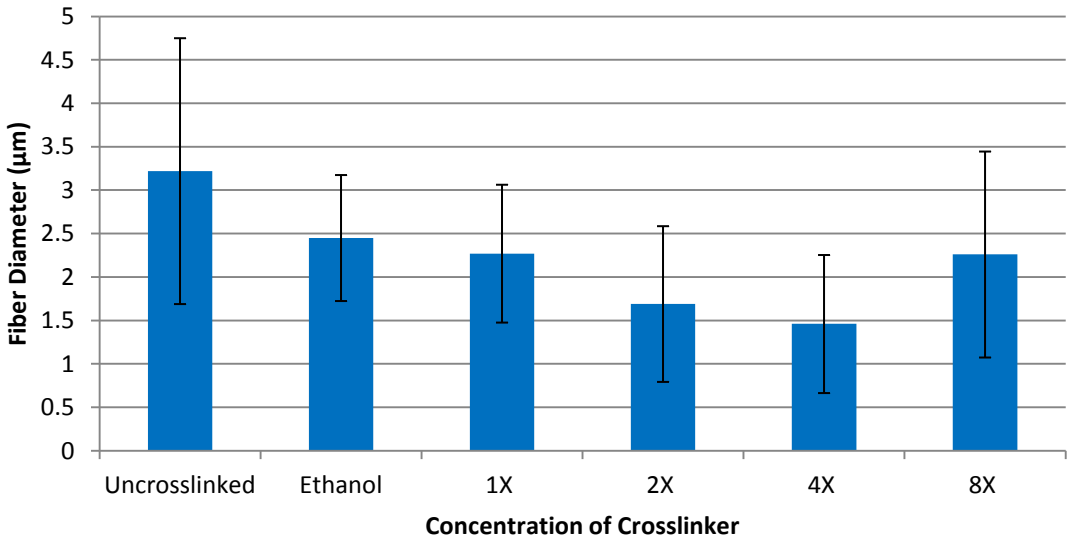


Figure 4.13: Fiber diameter measurements recorded from uncrosslinked and crosslinked electrospun gelatin nanofiber mats of varying concentrations of PEG-DA & DMPA. All crosslinked scaffolds except uncrosslinked control were incubated for 30 minutes before UV light treatment for two minutes on each side. All treated samples dried after crosslinking treatment.

4.2.3 Effect on tensile properties

Tensile testing was conducted to evaluate how gelatin nanofiber scaffolds mechanical properties were influenced when the crosslinker concentration was adjusted. Thickness, peak load, peak stress, modulus, strain at break and energy to break were all higher in ethanol samples compared to untreated samples. Crosslinked samples exhibited lower peak stress values from 17.48 MPa to 6.609 MPa as the concentrations of PEG-DA and DMPA were increased from 1X to 8X respectively. Elastic modulus also declined as a function of crosslinker concentration. 1X treated samples failed at 554.5 MPa and 8X treated samples failed at 155.5 MPa. 4X and 8X

scaffolds exhibited compromised elastic stiffness at mechanical failure. Also, the evaluated scaffolds with a higher concentration of crosslinking agents exhibited a lower fiber diameter and lower mechanical strength confirmed by previous work by Milleret et al.[50] 8X treated samples tested for strain at break exhibited significant statistical differences ($p<0.05$) when tested against all other subgroups. Eleven out of fifteen subgroup comparisons of crosslinker concentration tested for modulus showed no statistical differences. Nine out of fifteen subgroups comparisons of crosslinker concentration tested for peak load and peak stress also showed no statistical differences.

Table 4.7: Tensile properties as a function of crosslinker concentration.

Sample	Thickness (in)	Peak Load (N)	Peak Stress (MPa)	Modulus (MPa)	Strain At Break (mm/mm)	Energy to Break (N*mm)
Untreated	0.00915 ± 0.00120	1.062 ± 0.5374	1.759 ± 1.004	94.28 ± 41.41	0.0286 ± 0.0120	0.1045 ± 0.07620
Ethanol	0.0130 ± 0.00670	16.04± 6.791	18.36± 9.266	606.5± 201.9	0.0460± 0.0134	3.199±1.989
1X	0.0200 ± 0.00270	22.07 ± 5.416	17.48 ± 4.758	554.5 ± 153.8	0.0580 ± 0.0180	5.758 ± 1.842
2X	0.0220 ± 0.0104	16.61 ± 5.285	12.40 ± 5.126	383.8 ± 196.7	0.0520 ± 0.0094	3.775 ± 2.029
4X	0.0220 ± 0.0043	12.44 ± 1.922	8.715 ± 1.440	251.0 ± 53.63	0.0700 ± 0.0166	4.242 ± 1.111
8X	0.0300 ± 0.0029	13.50 ± 1.846	6.609 ± 1.080	155.5 ± 30.39	0.0950 ± 0.0230	5.941± 1.928

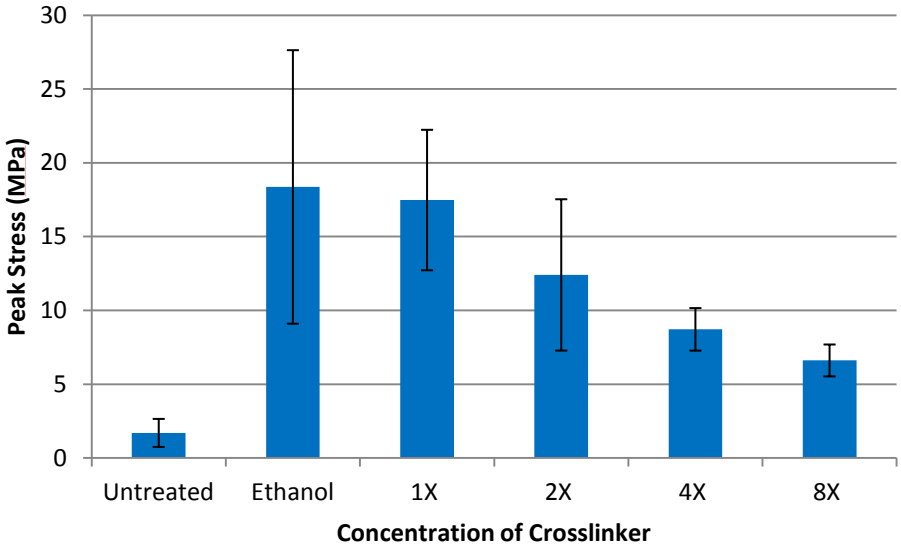


Figure 4.14: Peak stress measurements recorded from uncrosslinked and crosslinked electrospun gelatin nanofiber mats of varying concentrations of PEG-DA & DMPA. All crosslinked scaffolds except the uncrosslinked control were incubated for 30 minutes before UV light treatment for two minutes on each side. All treated samples dried after crosslinking treatment.

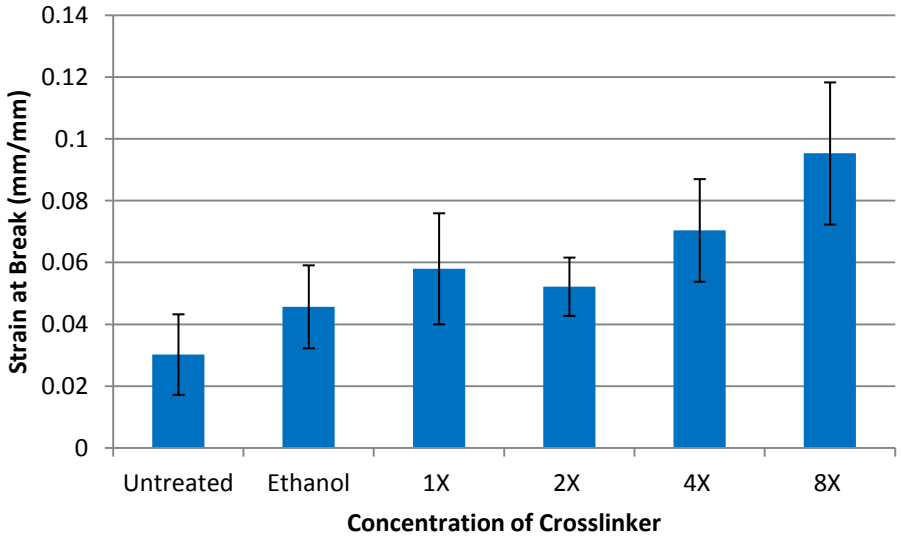


Figure 4.15: Strain at break measurements recorded from uncrosslinked and crosslinked electrospun gelatin nanofiber mats of varying concentrations of PEG-DA & DMPA. All crosslinked scaffolds except the uncrosslinked control were incubated for 30 minutes before UV light treatment for two minutes on each side. All treated samples dried after crosslinking treatment.

4.2.4 Effect on in vitro degradation

Degradation was significantly reduced by increasing crosslinker concentration. However, crosslinked scaffolds did not degrade appreciably between 6 h and 24 h. Scaffolds whose concentrations of PEG-DA and DMPA were doubled from 1X to 2X kept approximately 10% more weight over a 24 hour period. 4X crosslinked scaffolds kept 40% more weight than 1X samples. At the highest crosslinker concentration, 8X, scaffolds retained the majority of their weight, keeping approximately 70% of its weight over 24 hours. This direct relationship between crosslinker concentration and the degree of degradation indicated that gelatin nanofibers became increasingly stable to hydrolysis as crosslinker concentration was increased. Significant statistical differences ($p<0.05$) were shown for 1X concentrated scaffolds tested against 4X and 8X crosslinked scaffold subgroups.

Table 4.8: In vitro degradation in DMEM + 10% FBS as a function of crosslinker concentration.

Weight Loss in Sample	1X Concentration	2X Concentration	4X Concentration	8X Concentration
6 hours	100% ± 0.00	89.25% ± 5.360%	60.27% ± 2.010%	30.63% ± 4.780%
12 hours	100% ± 0.00	87.98% ± 2.870%	54.66% ± 4.630%	34.59% ± 2.200%
24 hours	97.14% ± 4.500%	89.74% ± 10.18%	61.25% ± 8.540%	33.41% ± 0.920%

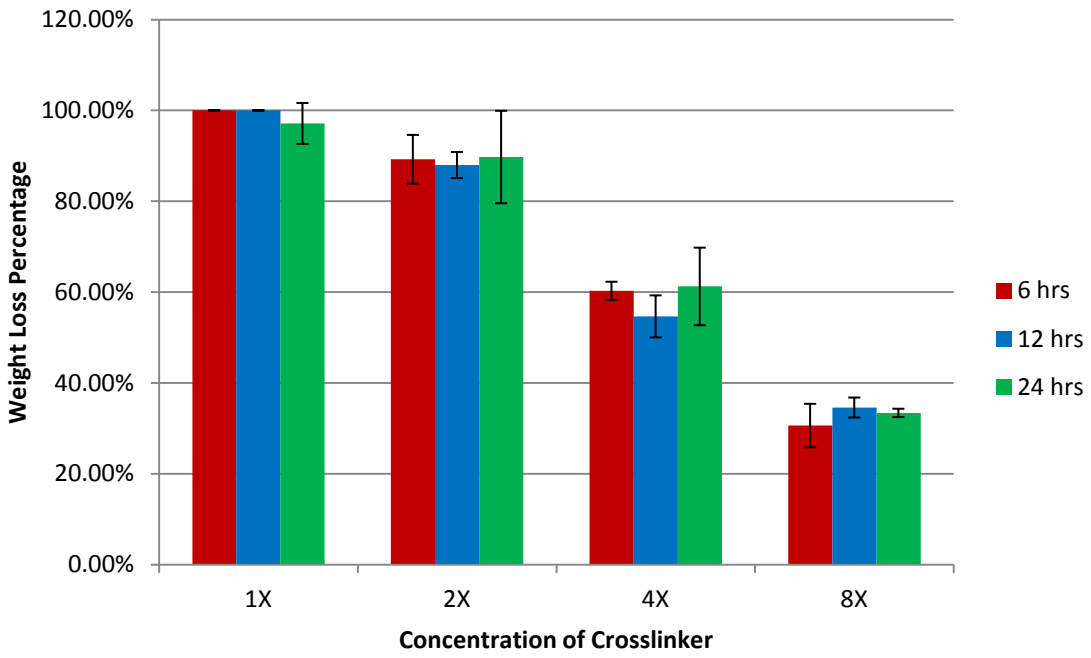


Figure 4.16: Degradation rate of crosslinked electrospun gelatin nanofiber mats with varying concentrations of PEG-DA & DMPA. All crosslinked scaffolds were then incubated for 30 minutes before UV light treatment for two minutes on each side. Scaffold degradation followed after immersion into DMEM + 10% fetal bovine serum. Degradation is calculated as $D(\%) = 100 \times (w_o - w_d)/w_o$, where w_o and w_e are the weight of the nanofibers before and after degradation at selected time points.

Table 4.9: In vitro degradation in SSF as a function of crosslinker concentration.

Weight Loss in Sample	1X Concentration	2X Concentration	4X Concentration	8X Concentration
6 hours	100% ± 0.00	86.31% ± 10.88%	82.31% ± 7.700%	56.75% ± 3.350%
12 hours	100% ± 0.00	88.55% ± 1.320%	77.22% ± 2.340%	58.82% ± 4.560%
24 hours	100% ± 0.00	69.10% ± 5.480%	79.54% ± 8.760%	61.12% ± 3.440%

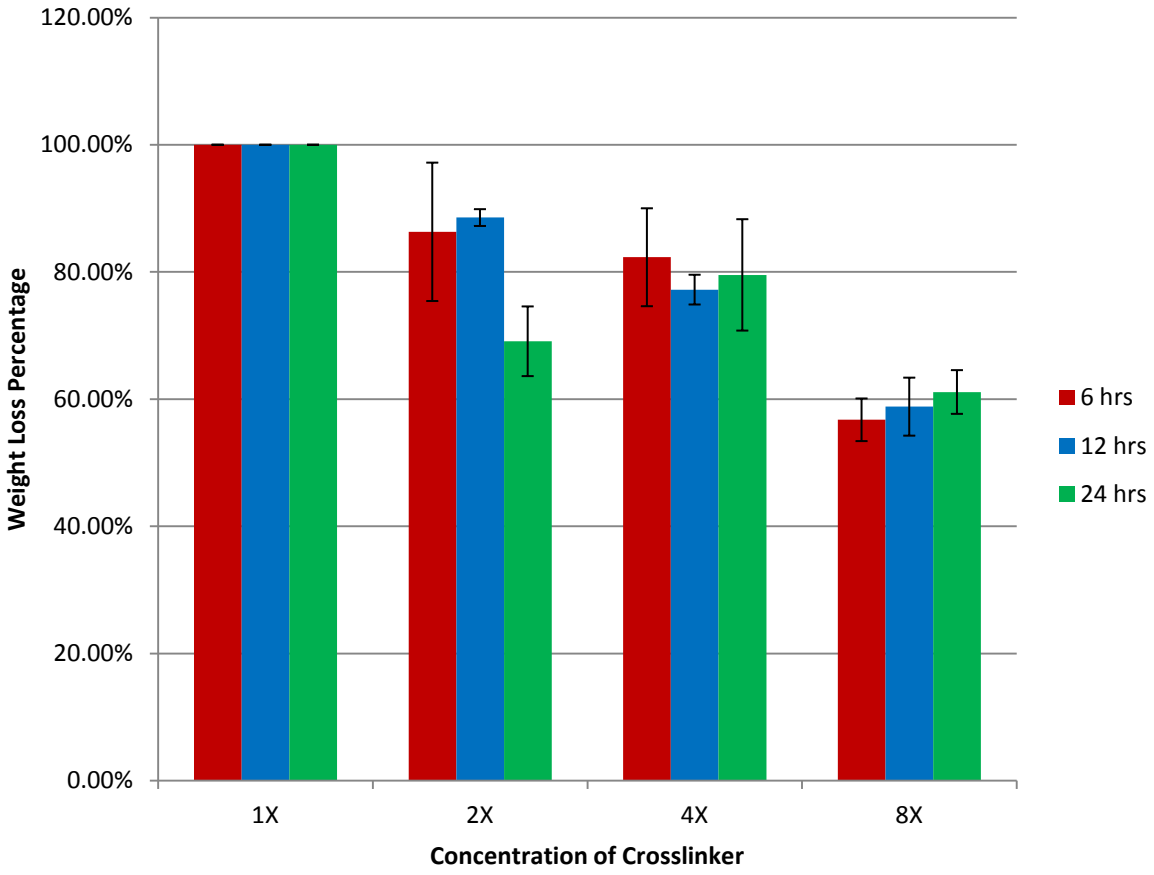


Figure 4.17: Degradation rate of crosslinked electrospun gelatin nanofiber mats with varying concentrations of PEG-DA & DMPA. All crosslinked scaffolds were then incubated for 30 minutes before UV light treatment for two minutes on each side. Scaffold degradation followed after immersion into simulated salivary fluid. Degradation is calculated as $D(\%) = 100 \times (w_o - w_d)/w_o$, where w_o and w_d are the weight of the nanofibers before and after degradation at selected time points.

Table 4.10: In vitro degradation in DMEM control as a function of crosslinker concentration.

Weight Loss in Sample	1X Concentration	2X Concentration	4X Concentration	8X Concentration
6 hours	93.69% ± 5.720%	65.96% ± 0.750%	41.55% ± 3.330%	16.94% ± 1.190%
12 hours	83.40% ± 1.450%	68.51% ± 4.430%	43.47% ± 2.050%	26.14% ± 4.390%
24 hours	96.80% ± 5.540%	65.39% ± 1.890%	45.36% ± 3.950%	24.49% ± 1.390%

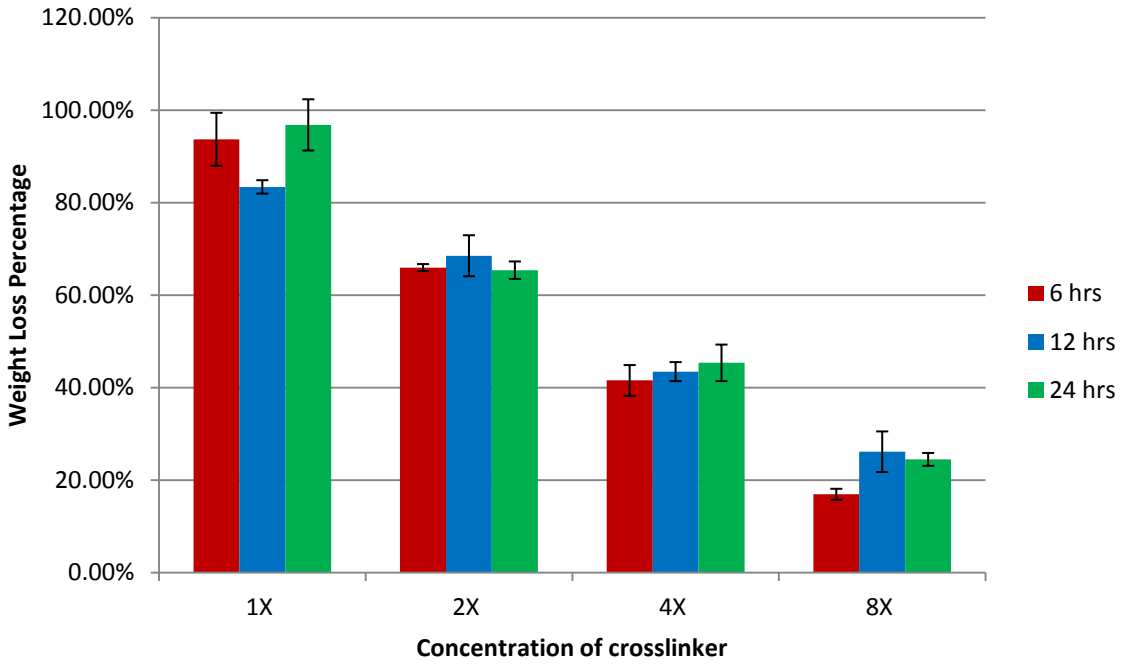


Figure 4.18: Degradation rate of crosslinked electrospun gelatin nanofiber mats with varying concentrations of PEG-DA & DMPA. All crosslinked scaffolds were then incubated for 30 minutes before UV light treatment for two minutes on each side. Scaffold degradation followed after immersion into DMEM. Degradation is calculated as $D(\%) = 100 \times (w_o - w_d)/w_o$, where w_o and w_d are the weight of the nanofibers before and after degradation at selected time points.

4.2.5 Effect on porosity

The degree of porosity of the crosslinked scaffolds decreased as crosslinker concentration increased. The untreated and ethanol controls exhibited average porosities of 82.15% and 84.58% respectively. Scaffolds crosslinked with 4X concentration of PEG-DA and DMPA had the lowest average porosity at 36.98%. Increasing crosslinker concentration reduced the fiber diameter size and porosity within the electrospun scaffold.[51] Additionally, the data confirmed that higher concentrations of PEG-DA and DMPA increased crosslinking and fiber interconnecting, leading to smaller diameters and lower porosities within the gelatin scaffold. There were significant differences ($p<0.05$) for ethanol and uncrosslinked treated samples tested against all subgroups except when they are evaluated versus each other.

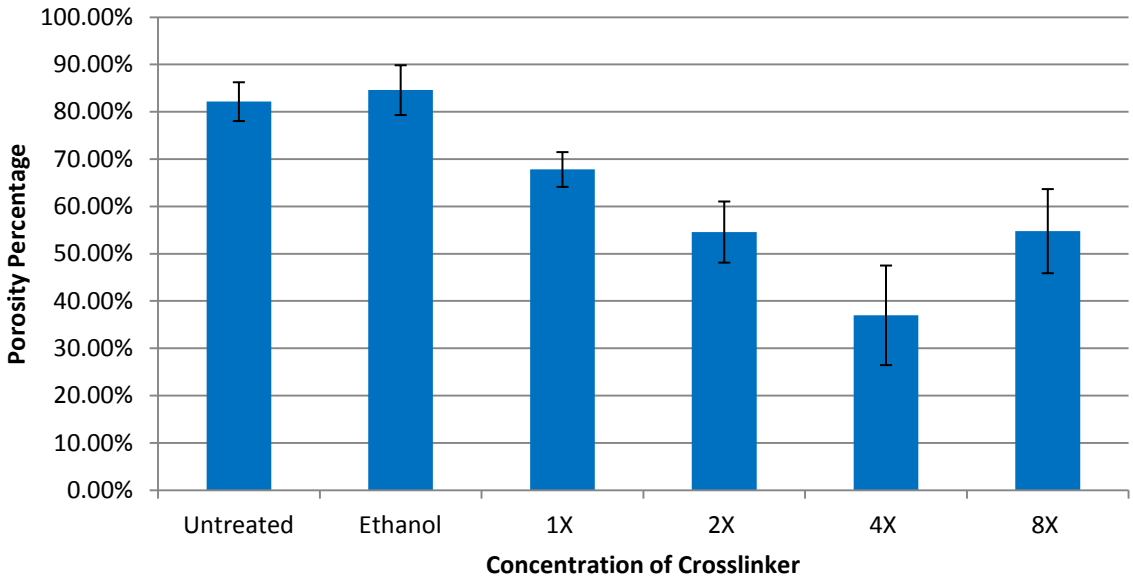


Figure 4.19: Porosity measurements recorded from uncrosslinked and crosslinked electrospun gelatin nanofiber mats with varying concentrations of PEG-DA & DMPA. All crosslinked scaffolds were then incubated for 30 minutes with two minutes of UV radiation on each side.

4.2.6 Effect on swelling kinetics

The lower crosslinked samples possessed elevated swelling rates but at the cost of high degradation. On the other hand, gelatin with the 8X concentration of PEG-DA and DMPA exhibited a swelling ratio five times lower than that of 1X (Figure 4.20) but was significantly more stable from degradation. The degree of swelling among crosslinked samples were varied based on the crosslinking agent concentration. Untreated, control and lower concentrated crosslinked samples (1X and 2X) exhibited significant burst swelling in the first 30 minutes. The high crosslinked scaffolds possessed a lower degree of burst swelling 30 minutes post-immersion in PBS. The lower degrees of swelling in the 4X and 8X samples were due to the presence of a denser crosslinked network. Those crosslinked networks were stable and resistant to water absorption as indicated in the degradation studies. Nine out of fifteen subgroups comparisons of crosslinker concentration tested for swelling showed no statistical differences.

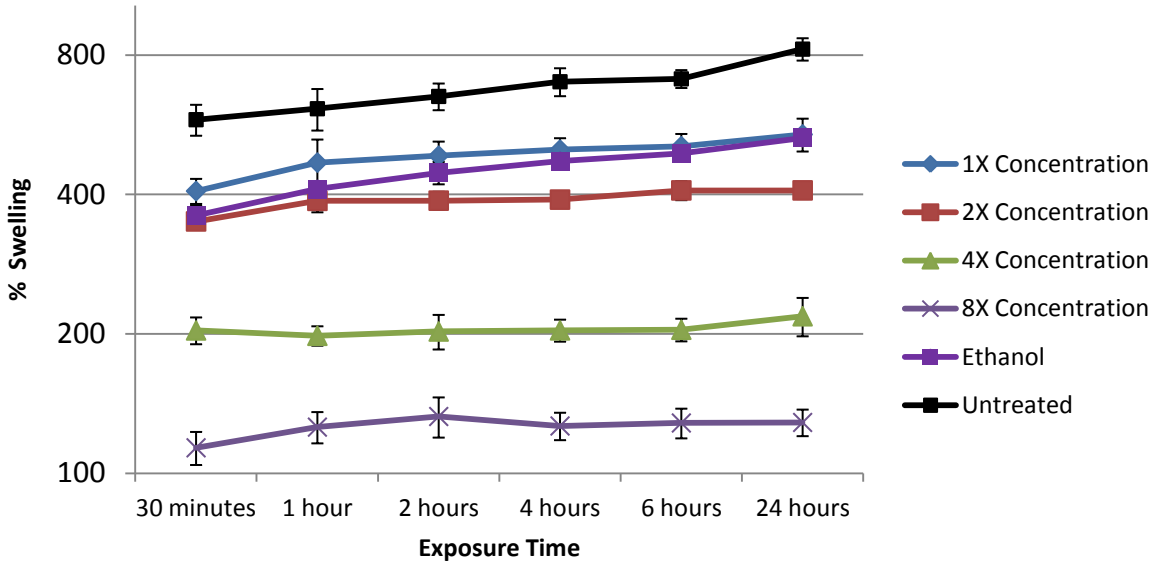


Figure 4.20: Swelling kinetics of crosslinked electrospun gelatin nanofiber mats of varying concentrations of PEG-DA & DMPA. All crosslinked scaffolds were then treated for 30 minutes times with two minutes of UV radiation on each side**.** Scaffold swelling followed after immersion into PBS at room temperature. Swelling is calculated as $S(\%) = 100 \times (m_o - m_e)/m_o$, where m_o and m_e are the weights of the dry and swollen nanofibers respectively at selected time points.

4.2.7 Comparison of mucoadhesion

Only 4X crosslinked scaffolds showed mucoadhesiveness. The 4X scaffold absorbed approximately 0.2 milligrams per square centimeter as shown on Figure 4.21. The absorption was attributed to 4X scaffolds possessing more flexible polymer chains that allowed water to diffuse into the matrix. The 4X scaffolds also promoted stability that prevented gelatin to dissolve and leak out into the surrounding solution. The 8X crosslinked nanofiber scaffold, 4X crosslinked hydrogel and 7.5% (w/v) PEG-1500 hydrogel exhibited negative absorption values. The 8X nanofiber scaffold contained an inflexible network, and more PEG chains, inhibiting diffusion of mucin and presenting a more resistant surface to protein adhesion. The 4X gelatin

crosslinked hydrogel control had poor mucoadhesion due to lack of porosity, preventing incorporation of mucin into the network. The 4X control hydrogel confirmed the nanofiber design may have had a positive effect on mucoadhesion. The 7.5% (w/v) PEG-1500 hydrogel had low mucoadhesion due to the non-fouling surface properties of PEG being inhibiting non-specific protein binding to the hydrogel surface. Significant statistical differences ($p<0.05$) were shown for 4X nanofiber scaffolds tested against other subgroups in the mucoadhesion study.

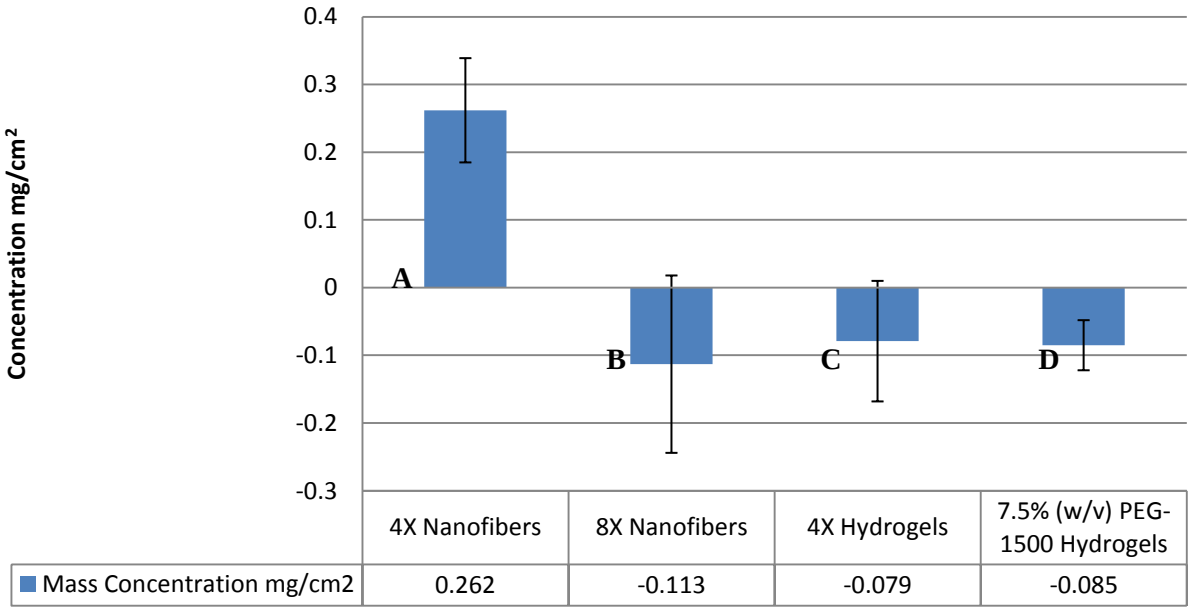


Figure 4.21: Cross-sectional mucoadhesion of A) 4X PEG-DA & DMPA crosslinked electrospun gelatin nanofibers, B) 8X PEG-DA & DMPA crosslinked electrospun gelatin nanofibers, C) 4X PEG-DA & DMPA crosslinked gelatin hydrogels, D) 7.5% (w/v) PEG-1500 hydrogels after immediate immersion in 1 mg/mL mucin solution.

4.2.8 Comparison of drug release kinetics in 4X scaffolds

4X crosslinked scaffolds were used in the drug release study for its good stability in aqueous conditions. Linear release kinetics was observed as in the first 24 hours but began to plateau out towards 120 hours. Scaffolds loaded with a low payload of nystatin had faster release

kinetics over 24 hour and 120 hour time periods. Scaffolds loaded with 50 and 100 mg of nystatin released 33.8 and 55.7% of its drug capacity in 24 hours respectively while 200 and 400 mg dosages released 32.1 and 25.1% of the total drug over 24 hours. The smaller dosages, 50 and 100 mg had a corresponding release of 57.1 and 74.1% of total drug after 120 hours. On the other hand, after 120 hours 43.3% of drug was released from the 200 mg dosage. The lower drug payload 4X scaffolds had faster release kinetics over the time interval because the smaller size of those scaffolds allowed for easier diffusion and faster degradation in the release medium whose volume was held constant. After 120 hours, the 400 mg dosage released only 35% of total drug, which was lowest among all the formulations. The release kinetics of the 50 and 100 dosage formulations were statistically different ($p<0.05$) when evaluated against other dosage formulation's release kinetics.

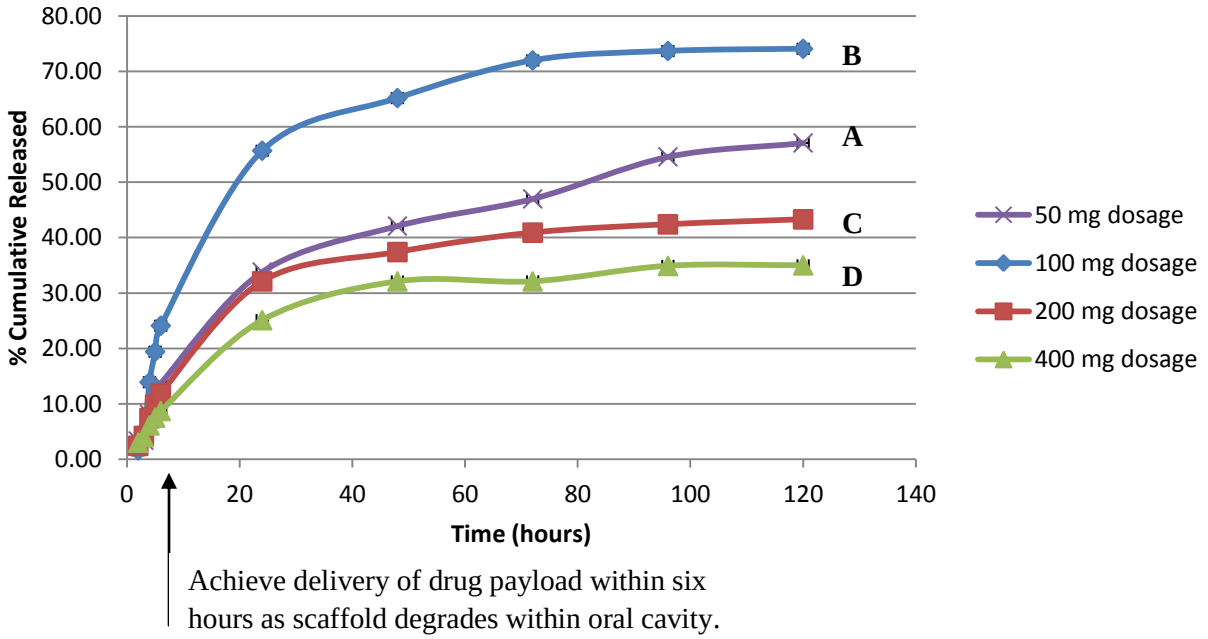


Figure 4.22: Cumulative release kinetics of varying amounts of nystatin: A) 50 mg, B) 100 mg, C) 200 mg and D) 400 mg loaded in electrospun gelatin nanofibers crosslinked with 4X PEG-DA & DMPA. Cumulative nystatin release was measured at predetermined timepoints.

CHAPTER 5: SUMMARY & FUTURE WORK

5.1 Summary

Electrospun nanofibers have been actively studied in tissue engineering scaffolding over the last decade. They have been explored for clinical applications in wound healing, orthopedics and cellular/tissue regeneration. However, nanofiber scaffolds as drug delivery vehicles within the oral cavity are relatively new. The effects of incubation time and concentration of PEG-diacrylate crosslinker on the structure and properties of crosslinked gelatin scaffolds were investigated. Additionally, the scaffold's mucoadhesive and drug release kinetics properties were tuned to ensure proper interfacing between the scaffold and oral mucosa for local and sustained nystatin drug delivery against oral candidiasis. The objective of this project was to find the most appropriate physical properties of the scaffold for time sensitive oral drug delivery within six hours of implantation. This was done by optimizing the crosslinking parameters with respect to incubation time and crosslinker concentration.

In summary, fiber morphology and tensile properties were retained regardless of incubation time or crosslinker concentration. Scaffold porosity was reduced as crosslinker concentration increased. In vitro degradation and swelling properties were influenced by the degree of porosity as well. Scaffolds with higher crosslinking densities of PEG-DA and greater incubation times had lower average porosities, inhibiting diffusion of media into the nanofiber gelatin matrix. Scaffolds with lower crosslinking densities and incubation time treatments had a greater area of pores within flexible nanofiber architectures to allow infusion of surrounding media into the matrix. As a result, higher crosslinked and longer incubation treated scaffolds possessed lower swelling ratios and in vitro degradation at room and body temperatures

respectively. Scaffolds with a lower crosslinking density and incubation time treatments had higher swelling ratios and in vitro degradation in the same conditions. The increased temperature in the degradation study conditions caused the gelatin nanofibers to liquefy and dissolve due to its transition phase from solid to solution at 37°C.[52,53] As shown in Table 5.1, it appears that the 30 minute incubated and 1X concentrated scaffolds possessed the best physical properties for oral drug delivery. However, its poor stability and lack of resistance to degradation does not allow the particular scaffold to deliver a constant drug dosage within six hours due to its relative fast dissolution in high temperature aqueous media. The 8X crosslinked scaffolds also possessed good physical characteristics also shown in Table 5.1. These scaffolds were not clinically feasible due to its high stability in aqueous solutions, causing the material to hang around in the patient's oral cavity beyond the six hour timeframe, leading to compliance and practicality issues. Gelatin scaffolds with 4X crosslinking concentrations were chosen for mucoadhesion and drug release studies in place of the 2X scaffolds because of its greater relative stability while possessing enough amorphous properties to dissolve in aqueous solution within six hours.

After physical characterization, 4X concentrated scaffolds were evaluated to determine a suitable combination of crosslinker concentration and drug dosage that would produce a nanofiber scaffold with good mucoadhesion and controlled drug release properties. The mucoadhesion and drug release studies showed that 4X crosslinked gelatin nanofiber scaffolds containing 50 and 100 mg nystatin dosages have the capability to work as a bioadhesive patch for controlled and efficient drug delivery for oral candidiasis treatment. Yet, the drug payload within the targeted first six hours needs to be significantly improved from the 25% cumulative release shown for 100 mg dosage loaded samples in the nystatin kinetics study.

Table 5.1: Summary table

Properties	30 minute Incubation	12 hours Incubation	24 hours Incubation	1X Concentration	2X Concentration	4X Concentration	8X Concentration
Fiber Morphology	✓	✓	✓	✓	✓	✓	✓
Fiber Diameter	✓✓	✓✓	✓	✓✓	✓	✓	✓✓
Tensile Properties	✓✓	✓	✓	✓✓	✓	✓	✓
Degradation Resistance	NA	✓	✓	NA	✓	✓✓	✓✓✓
Porosity	✓✓✓	✓	✓✓	✓✓✓	✓✓	✓	✓✓
Swelling	✓✓✓	✓	✓	✓✓✓	✓✓	✓	✓

5.2 Future work

Stable electrospun nanofiber scaffolds are novel formulations for drug delivery within the oral cavity, helping to improve bioavailabity and extend residence time for local sustained drug release in oral candidiasis treatment. To evaluate the efficacy of the nystatin loaded scaffold, in vitro anti-microbial tests should be conducted using nystatin loaded scaffolds on cultured fungal cell lines. Biocompatibility and cell viability studies using oral epithelium cells will need to be conducted to ensure the formulation not only effectively treats candidiasis but is also safe for patients. More testing and improvements in mucoadhesion, drug release and degradation studies are necessary in the future to achieve clinical feasibility. Direct mucoadhesion tests in different physiological environments (i.e. pH and flow rate) need to be done to evaluate the adhesion of gelatin nanofiber scaffolds onto the oral mucosa. In vitro tests including tensile detachment testing, rheology, rotating cylinder or epithelial cell layer models would be applied to give a concrete analysis of the scaffold's bioadhesion capabilities.[54] Integrating an adhesive polymer backing on the scaffold would also ensure proper adhesion although direction of the drug release from the multi-layered scaffold will also have to be regarded. Drug release studies will be further

examined using 2X crosslinked scaffolds to determine if crosslinker concentration and dosage level have an influence on nystatin release kinetics. This drug release study can help

determine if the 2X scaffold formulation can achieve targeted degradation and greater dosage release within the first six hours. In vitro degradation testing will also need to be done using aqueous media that better reflects the salivary conditions within the oral cavity. Introducing mucin proteins, electrolytes such as bicarbonate and magnesium and enzymes like amylase, lysozymes and matrix metalloproteinases (MMPs) would improve simulating saliva conditions within the oral cavity.[55] Incorporating these salivary components to the existing simulated salivary fluid (SSF) formulation will give a much more accurate physical evaluation of how crosslinked gelatin nanofibers interact in saliva. Future in vitro degradation, swelling and drug release kinetics testing need to be done using nanofiber gelatin scaffolds immersed in improved simulated salivary fluid conditions.

There are still existing limitations for the practicality of this formulation that will need to be rectified in future projects. The physiological environment of the oral cavity introduces challenges of drug delivery because of the constant flow of saliva diluting and wash out of drug dosages below therapeutic levels, shortening its residence time. The relative small area of the buccal mucosa also restricts absorption time and dosage size of the drug loaded scaffold.[56] Metalloproteinase enzymes in saliva that function by degrading extracellular matrix materials need to be taken into account in designing scaffolds with six hour targeted degradation and drug release. Evaluating the physical properties of the scaffold in diseased and normal physiological conditions in the oral mucosa would also help validate the clinical efficacy of this formulation. Lastly, improving material processing, crosslinking methods and exploring other therapeutics for safe and effective oral mucosa drug delivery will be considered in future studies.

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Appendix A

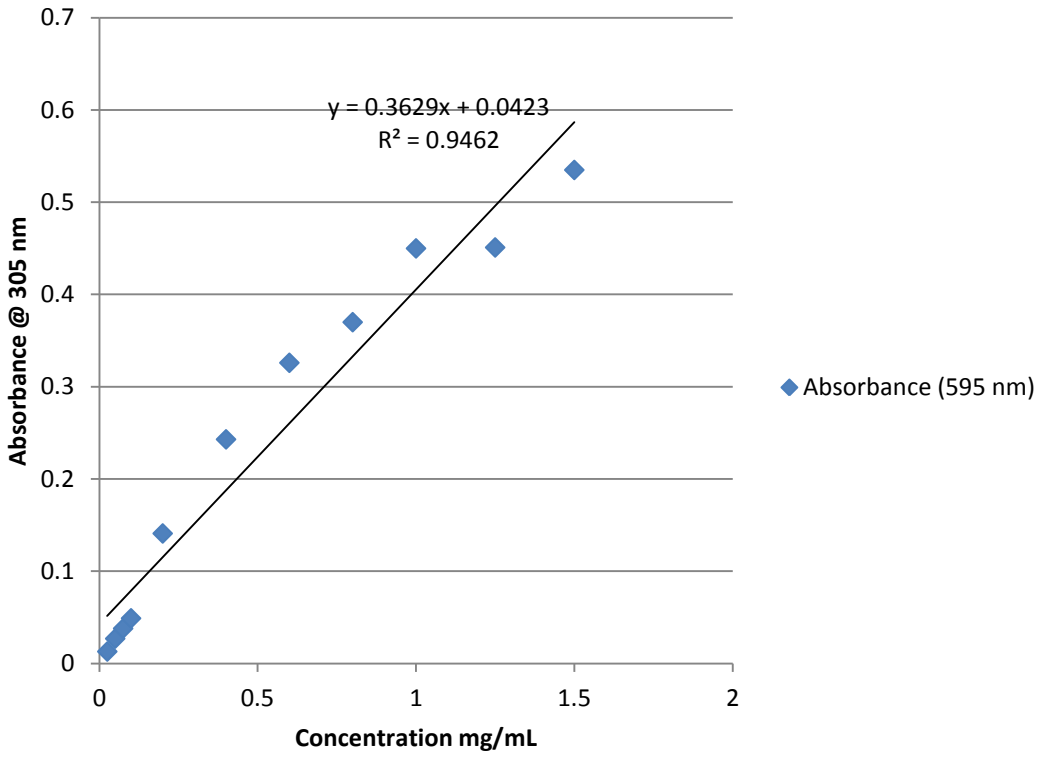

Figure A.1: Mucin standard curve

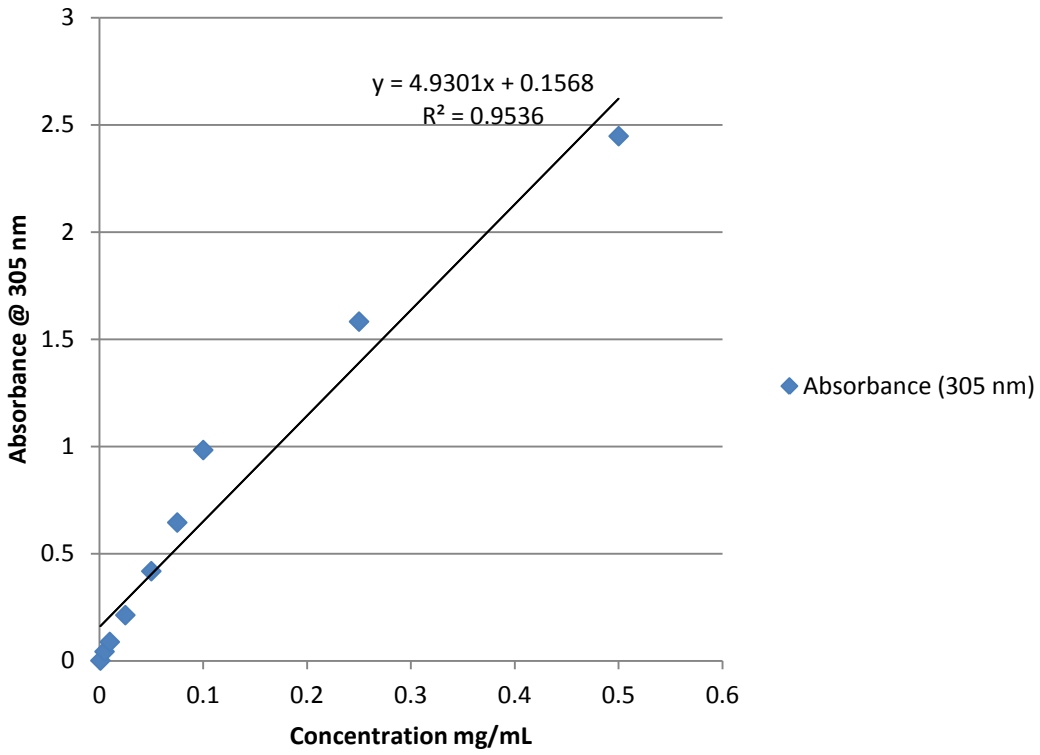

Figure A.2: Nystatin standard curve

Appendix B

Interpretation of Statistical Analysis

Analysis of Variance (ANOVA):

- $p < 0.05$ indicates that the data is statistically significant

Tukey's Pairwise Comparisons:

- Tukey's pairwise comparison is performed to determine if two data values is statistically different from one another

One Way Analysis of Variance ---Fiber Diameter as a function of incubation time (Figure 4.3)

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Failed ($P < 0.050$)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Data source: Data 1 in Notebook1

Group	N	Missing	Median	25%	75%
Uncrosslinked	60	0	3.175	1.992	4.332
Ethanol	60	0	2.540	2.060	2.873
30 minute incubation (1X)	60	0	2.160	1.725	2.740
12 hour incubation (1X)	60	0	2.340	1.763	2.768
24 hour incubation (1X)	60	0	1.500	1.060	2.202

H = 50.584 with 4 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	p<0.05
Uncrosslinked vs 24 hour incub	6593.500	9.813	Yes
Uncrosslinked vs 30 minute inc	3445.000	5.127	Yes
Uncrosslinked vs 12 hour incub	3051.000	4.541	Yes
Uncrosslinked vs Ethanol	2143.000	3.189	No
Ethanol vs 24 hour incub	4450.500	6.623	Yes
Ethanol vs 30 minute inc	1302.000	1.938	No
Ethanol vs 12 hour incub	908.000	1.351	Do Not Test
12 hour incub vs 24 hour incub	3542.500	5.272	Yes
12 hour incub vs 30 minute inc	394.000	0.586	Do Not Test
30 minute inc vs 24 hour incub	3148.500	4.686	Yes

Note: The multiple comparisons on ranks do not include an adjustment for ties.

A result of "Do Not Test" occurs for a comparison when no significant difference is found between the two rank sums that enclose that comparison. For example, if you had four rank sums sorted in order, and found no significant difference between rank sums 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed rank sums is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the rank sums, even though one may appear to exist.

One Way Analysis of Variance—Fiber Diameter as a function of Crosslinker concentration (Figure 4.13)

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Data source: Data 1 in Notebook1

Group	N	Missing	Median	25%	75%
Uncrosslinked	60	0	3.175	1.992	4.332
Ethanol	60	0	2.540	2.060	2.873
1X Concentration	60	0	2.160	1.725	2.740
2X Concentration	60	0	1.540	0.925	2.212
4X Concentration	60	0	1.215	0.823	2.138
8X Concentration	60	0	2.015	1.385	3.435

H = 78.135 with 5 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	p<0.05
Uncrosslinked vs 4X Concentrat	8545.000	10.600	Yes
Uncrosslinked vs 2X Concentrat	7242.500	8.985	Yes
Uncrosslinked vs 8X Concentrat	4156.500	5.156	Yes
Uncrosslinked vs 1X Concentrat	3434.000	4.260	Yes
Uncrosslinked vs Ethanol	2098.000	2.603	No
Ethanol vs 4X Concentration	6447.000	7.998	Yes
Ethanol vs 2X Concentration	5144.500	6.382	Yes
Ethanol vs 8X Concentration	2058.500	2.554	No
Ethanol vs 1X Concentration	1336.000	1.657	Do Not Test
1X Concentrat vs 4X Concentrat	5111.000	6.340	Yes
1X Concentrat vs 2X Concentrat	3808.500	4.725	Yes
1X Concentrat vs 8X Concentrat	722.500	0.896	Do Not Test
8X Concentrat vs 4X Concentrat	4388.500	5.444	Yes
8X Concentrat vs 2X Concentrat	3086.000	3.828	No
2X Concentrat vs 4X Concentrat	1302.500	1.616	No

Note: The multiple comparisons on ranks do not include an adjustment for ties.

A result of "Do Not Test" occurs for a comparison when no significant difference is found between the two rank sums that enclose that comparison. For example, if you had four rank sums sorted in order, and found no significant difference between rank sums 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed rank sums is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the rank sums, even though one may appear to exist.

One Way Analysis of Variance--Thickness as a function of incubation time (Table 4.2)

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Data source: Data 1 in Notebook1

Group	N	Missing	Median	25%	75%
Uncrosslinked	10	0	0.00900	0.00850	0.01000
Ethanol	10	0	0.0130	0.0109	0.0145
30 minute incubation (1X)	10	0	0.0180	0.0140	0.0266
12 hour incubation (1X)	10	0	0.0138	0.0124	0.0154
24 hour incubation (1X)	10	0	0.0292	0.0181	0.0386

H = 30.752 with 4 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	p<0.05
24 hour incub vs Uncrosslinked	330.500	7.170	Yes
24 hour incub vs Ethanol	162.000	3.514	No
24 hour incub vs 12 hour incub	131.500	2.853	Do Not Test
24 hour incub vs 30 minute inc	46.000	0.998	Do Not Test
30 minute inc vs Uncrosslinked	284.500	6.172	Yes
30 minute inc vs Ethanol	116.000	2.516	Do Not Test
30 minute inc vs 12 hour incub	85.500	1.855	Do Not Test
12 hour incub vs Uncrosslinked	199.000	4.317	Yes
12 hour incub vs Ethanol	30.500	0.662	Do Not Test
Ethanol vs Uncrosslinked	168.500	3.655	No

Note: The multiple comparisons on ranks do not include an adjustment for ties.

A result of "Do Not Test" occurs for a comparison when no significant difference is found between the two rank sums that enclose that comparison. For example, if you had four rank sums sorted in order, and found no significant difference between rank sums 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4

vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed rank sums is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the rank sums, even though one may appear to exist.

One Way Analysis of Variance—Thickness as a function of Crosslinker concentration (Table 4.7)

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Passed (P = 0.131)

Equal Variance Test: Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Data source: Data 1 in Notebook1

Group	N	Missing	Median	25%	75%
Uncrosslinked	10	0	0.00900	0.00850	0.01000
Ethanol	10	0	0.0130	0.0109	0.0145
1X Concentration	10	0	0.0180	0.0140	0.0266
2X Concentration	10	0	0.0220	0.0125	0.0334
4X Concentration	10	0	0.0200	0.0174	0.0251
8X Concentration	10	0	0.0290	0.0278	0.0319

H = 37.965 with 5 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	p<0.05
8X Concentrat vs Uncrosslinked	442.500	8.012	Yes
8X Concentration vs Ethanol	304.500	5.514	Yes
8X Concentrat vs 1X Concentrat	177.500	3.214	No
8X Concentrat vs 2X Concentrat	155.000	2.807	Do Not Test
8X Concentrat vs 4X Concentrat	141.500	2.562	Do Not Test
4X Concentrat vs Uncrosslinked	301.000	5.450	Yes
4X Concentration vs Ethanol	163.000	2.951	No
4X Concentrat vs 1X Concentrat	36.000	0.652	Do Not Test
4X Concentrat vs 2X Concentrat	13.500	0.244	Do Not Test
2X Concentrat vs Uncrosslinked	287.500	5.206	Yes
2X Concentration vs Ethanol	149.500	2.707	Do Not Test
2X Concentrat vs 1X Concentrat	22.500	0.407	Do Not Test
1X Concentrat vs Uncrosslinked	265.000	4.798	Yes
1X Concentration vs Ethanol	127.000	2.300	Do Not Test
Ethanol vs Uncrosslinked	138.000	2.499	No

Note: The multiple comparisons on ranks do not include an adjustment for ties.

A result of "Do Not Test" occurs for a comparison when no significant difference is found between the two rank sums that enclose that comparison. For example, if you had four rank sums sorted in order, and found no significant difference between rank sums 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed rank sums is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the rank sums, even though one may appear to exist.

One Way Analysis of Variance--Peak Load as a function of incubation time (Table 4.2)

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Passed (P = 0.166)

Equal Variance Test: Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Data source: Data 1 in Notebook1

Group	N	Missing	Median	25%	75%
Uncrosslinked	10	0	0.889	0.537	1.614
Ethanol	10	0	14.601	11.171	21.648
30 minute incubation (1X)	10	0	21.970	16.517	27.424
12 hour incubation (1X)	10	0	15.623	14.462	18.215
24 hour incubation (1X)	10	0	17.458	14.843	26.029

H = 27.825 with 4 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	p<0.05
30 minute inc vs Uncrosslinked	322.000	6.985	Yes
30 minute inc vs Ethanol	120.000	2.603	No
30 minute inc vs 12 hour incub	110.000	2.386	Do Not Test
30 minute inc vs 24 hour incub	58.000	1.258	Do Not Test
24 hour incub vs Uncrosslinked	264.000	5.727	Yes
24 hour incub vs Ethanol	62.000	1.345	Do Not Test
24 hour incub vs 12 hour incub	52.000	1.128	Do Not Test
12 hour incub vs Uncrosslinked	212.000	4.599	Yes
12 hour incub vs Ethanol	10.000	0.217	Do Not Test
Ethanol vs Uncrosslinked	202.000	4.382	Yes

Note: The multiple comparisons on ranks do not include an adjustment for ties.

A result of "Do Not Test" occurs for a comparison when no significant difference is found between the two rank sums that enclose that comparison. For example, if you had four rank sums sorted in order, and found no significant difference between rank sums 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed rank sums is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the rank sums, even though one may appear to exist.

One Way Analysis of Variance--Peak Load as a function of Crosslinker concentration (Table 4.7)

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Data source: Data 1 in Notebook1

Group	N	Missing	Median	25%	75%
Uncrosslinked	10	0	0.889	0.537	1.614
Ethanol	10	0	14.601	11.171	21.648
1X Concentration	10	0	21.970	16.517	27.424
2X Concentration	10	0	15.316	12.929	20.174
4X Concentration	10	0	12.177	10.554	13.677
8X Concentration	10	0	14.235	11.511	15.220

H = 36.665 with 5 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	p<0.05
1X Concentrat vs Uncrosslinked	449.000	8.130	Yes
1X Concentrat vs 4X Concentrat	254.000	4.599	Yes
1X Concentrat vs 8X Concentrat	206.000	3.730	No
1X Concentration vs Ethanol	154.000	2.789	Do Not Test
1X Concentrat vs 2X Concentrat	131.000	2.372	Do Not Test
2X Concentrat vs Uncrosslinked	318.000	5.758	Yes
2X Concentrat vs 4X Concentrat	123.000	2.227	No
2X Concentrat vs 8X Concentrat	75.000	1.358	Do Not Test
2X Concentration vs Ethanol	23.000	0.416	Do Not Test
Ethanol vs Uncrosslinked	295.000	5.342	Yes
Ethanol vs 4X Concentration	100.000	1.811	Do Not Test
Ethanol vs 8X Concentration	52.000	0.942	Do Not Test
8X Concentrat vs Uncrosslinked	243.000	4.400	Yes
8X Concentrat vs 4X Concentrat	48.000	0.869	Do Not Test
4X Concentrat vs Uncrosslinked	195.000	3.531	No

Note: The multiple comparisons on ranks do not include an adjustment for ties.

A result of "Do Not Test" occurs for a comparison when no significant difference is found between the two rank sums that enclose that comparison. For example, if you had four rank sums sorted in order, and found no significant difference between rank sums 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed rank sums is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the rank sums, even though one may appear to exist.

One Way Analysis of Variance--Peak Stress as a function of incubation time (Figure 4.4)

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Data source: Data 1 in Notebook1

Group	N	Missing	Median	25%	75%
Uncrosslinked	10	0	1.459	0.836	2.425
Ethanol	10	0	17.517	11.653	22.595
30 minute incubation (1X)	10	0	17.253	13.560	22.368
12 hour incubation (1X)	10	0	18.327	14.544	20.211
24 hour incubation (1X)	10	0	10.216	8.272	13.457

H = 29.270 with 4 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	p<0.05
12 hour incub vs Uncrosslinked	290.000	6.291	Yes
12 hour incub vs 24 hour incub	135.000	2.929	No
12 hour incub vs Ethanol	18.000	0.390	Do Not Test
12 hour incub vs 30 minute inc	7.000	0.152	Do Not Test
30 minute inc vs Uncrosslinked	283.000	6.139	Yes
30 minute inc vs 24 hour incub	128.000	2.777	Do Not Test
30 minute inc vs Ethanol	11.000	0.239	Do Not Test
Ethanol vs Uncrosslinked	272.000	5.901	Yes
Ethanol vs 24 hour incub	117.000	2.538	Do Not Test
24 hour incub vs Uncrosslinked	155.000	3.362	No

Note: The multiple comparisons on ranks do not include an adjustment for ties.

A result of "Do Not Test" occurs for a comparison when no significant difference is found between the two rank sums that enclose that comparison. For example, if you had four rank sums sorted in order, and found no significant difference between rank sums 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4

vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed rank sums is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the rank sums, even though one may appear to exist.

One Way Analysis of Variance--Peak Stress as a function of Crosslinker concentration (Figure 4.14)

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Data source: Data 1 in Notebook1

Group	N	Missing	Median	25%	75%
Uncrosslinked	10	0	1.459	0.836	2.425
Ethanol	10	0	17.517	11.653	22.595
1X Concentration	10	0	17.253	13.560	22.368
2X Concentration	10	0	10.858	9.144	17.506
4X Concentration	10	0	8.404	7.943	10.223
8X Concentration	10	0	6.445	5.972	7.486

H = 44.664 with 5 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	p<0.05
1X Concentrat vs Uncrosslinked	422.500	7.650	Yes
1X Concentrat vs 8X Concentrat	292.500	5.296	Yes
1X Concentrat vs 4X Concentrat	197.500	3.576	No
1X Concentrat vs 2X Concentrat	107.000	1.937	Do Not Test
1X Concentration vs Ethanol	15.500	0.281	Do Not Test
Ethanol vs Uncrosslinked	407.000	7.370	Yes
Ethanol vs 8X Concentration	277.000	5.016	Yes
Ethanol vs 4X Concentration	182.000	3.296	Do Not Test
Ethanol vs 2X Concentration	91.500	1.657	Do Not Test
2X Concentrat vs Uncrosslinked	315.500	5.713	Yes
2X Concentrat vs 8X Concentrat	185.500	3.359	No
2X Concentrat vs 4X Concentrat	90.500	1.639	Do Not Test
4X Concentrat vs Uncrosslinked	225.000	4.074	Yes
4X Concentrat vs 8X Concentrat	95.000	1.720	Do Not Test
8X Concentrat vs Uncrosslinked	130.000	2.354	No

Note: The multiple comparisons on ranks do not include an adjustment for ties.

A result of "Do Not Test" occurs for a comparison when no significant difference is found between the two rank sums that enclose that comparison. For example, if you had four rank sums sorted in order, and found no significant difference between rank sums 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed rank sums is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the rank sums, even though one may appear to exist.

One Way Analysis of Variance--Modulus as a function of incubation time (Table 4.2)

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Passed (P = 0.191)

Equal Variance Test: Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Data source: Data 1 in Notebook1

Group	N	Missing	Median	25%	75%
Uncrosslinked	10	0	77.049	67.471	116.602
Ethanol	10	0	603.821	414.401	743.849
30 minute incubation (1X)	10	0	569.202	409.878	684.755
12 hour incubation (1X)	10	0	535.358	415.855	593.311
24 hour incubation (1X)	10	0	292.010	178.048	527.663

H = 27.950 with 4 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	p<0.05
Ethanol vs Uncrosslinked	293.000	6.356	Yes
Ethanol vs 24 hour incub	134.000	2.907	No
Ethanol vs 12 hour incub	38.000	0.824	Do Not Test
Ethanol vs 30 minute inc	15.000	0.325	Do Not Test
30 minute inc vs Uncrosslinked	278.000	6.031	Yes
30 minute inc vs 24 hour incub	119.000	2.581	Do Not Test
30 minute inc vs 12 hour incub	23.000	0.499	Do Not Test
12 hour incub vs Uncrosslinked	255.000	5.532	Yes
12 hour incub vs 24 hour incub	96.000	2.083	Do Not Test
24 hour incub vs Uncrosslinked	159.000	3.449	No

Note: The multiple comparisons on ranks do not include an adjustment for ties.

A result of "Do Not Test" occurs for a comparison when no significant difference is found between the two rank sums that enclose that comparison. For example, if you had four rank sums sorted in order, and found no significant difference between rank sums 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed rank sums is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the rank sums, even though one may appear to exist.

One Way Analysis of Variance--Modulus as a function of Crosslinker concentration (Table 4.7)

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Data source: Data 1 in Notebook1

Group	N	Missing	Median	25%	75%
Uncrosslinked	10	0	77.049	67.471	116.602
Ethanol	10	0	603.821	414.401	743.849
1X Concentration	10	0	569.202	409.878	684.755
2X Concentration	10	0	336.157	201.922	569.997
4X Concentration	10	0	230.159	212.106	294.247
8X Concentration	10	0	163.246	136.662	179.930

H = 46.305 with 5 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	p<0.05
Ethanol vs Uncrosslinked	416.000	7.533	Yes
Ethanol vs 8X Concentration	332.000	6.012	Yes
Ethanol vs 4X Concentration	197.000	3.567	No
Ethanol vs 2X Concentration	133.000	2.408	Do Not Test
Ethanol vs 1X Concentration	14.000	0.254	Do Not Test
1X Concentrat vs Uncrosslinked	402.000	7.279	Yes
1X Concentrat vs 8X Concentrat	318.000	5.758	Yes
1X Concentrat vs 4X Concentrat	183.000	3.314	Do Not Test
1X Concentrat vs 2X Concentrat	119.000	2.155	Do Not Test
2X Concentrat vs Uncrosslinked	283.000	5.124	Yes
2X Concentrat vs 8X Concentrat	199.000	3.603	No
2X Concentrat vs 4X Concentrat	64.000	1.159	Do Not Test
4X Concentrat vs Uncrosslinked	219.000	3.965	No
4X Concentrat vs 8X Concentrat	135.000	2.444	Do Not Test
8X Concentrat vs Uncrosslinked	84.000	1.521	Do Not Test

Note: The multiple comparisons on ranks do not include an adjustment for ties.

A result of "Do Not Test" occurs for a comparison when no significant difference is found between the two rank sums that enclose that comparison. For example, if you had four rank sums sorted in order, and found no significant difference between rank sums 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed rank sums is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the rank sums, even though one may appear to exist.

One Way Analysis of Variance--Strain at Break as a function of incubation time (Figure 4.5)

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Passed (P = 0.129)

Equal Variance Test: Passed (P = 0.456)

Group Name	N	Missing	Mean	Std Dev	SEM
Uncrosslinked	10	0	0.0286	0.0120	0.00380
Ethanol	10	0	0.0457	0.0134	0.00424
30 minute incubation (1X)	10	0	0.0580	0.0180	0.00568
12 hour incubation (1X)	10	0	0.0588	0.0200	0.00632
24 hour incubation (1X)	10	0	0.0557	0.0181	0.00573

Source of Variation	DF	SS	MS	F	P
Between Groups	4	0.00648	0.00162	5.897	<0.001
Residual	45	0.0124	0.000275		
Total	49	0.0189			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 0.946

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	p<0.050
12 hour incu vs. Uncrosslinke	0.0302	5	5.760	0.002	Yes
12 hour incu vs. Ethanol	0.0131	5	2.499	0.405	No
12 hour incu vs. 24 hour incu	0.00310	5	0.591	0.993	Do Not Test
12 hour incu vs. 30 minute in	0.000800	5	0.153	1.000	Do Not Test
30 minute in vs. Uncrosslinke	0.0294	5	5.608	0.002	Yes
30 minute in vs. Ethanol	0.0123	5	2.346	0.469	Do Not Test
30 minute in vs. 24 hour incu	0.00230	5	0.439	0.998	Do Not Test
24 hour incu vs. Uncrosslinke	0.0271	5	5.169	0.006	Yes
24 hour incu vs. Ethanol	0.01000	5	1.907	0.663	Do Not Test
Ethanol vs. Uncrosslinked	0.0171	5	3.262	0.162	No

A result of "Do Not Test" occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed means is a procedural rule, and a result of Do Not

Test should be treated as if there is no significant difference between the means, even though one may appear to exist.

One Way Analysis of Variance--Strain at Break as a function of Crosslinker concentration

(Figure 4.15)

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Passed (P = 0.307)

Equal Variance Test: Passed (P = 0.170)

Group Name	N	Missing	Mean	Std Dev	SEM
Uncrosslinked	10	0	0.0286	0.0120	0.00380
Ethanol	10	0	0.0457	0.0134	0.00424
1X Concentration	10	0	0.0580	0.0180	0.00568
2X Concentration	10	0	0.0522	0.00943	0.00298
4X Concentration	10	0	0.0704	0.0166	0.00525
8X Concentration	10	0	0.0953	0.0230	0.00727

Source of Variation	DF	SS	MS	F	P
Between Groups	5	0.0259	0.00519	20.214	<0.001
Residual	54	0.0139	0.000257		
Total	59	0.0398			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	p<0.050
8X Concentra vs. Uncrosslinke	0.0667	6	13.167	<0.001	Yes
8X Concentration vs. Ethanol	0.0496	6	9.792	<0.001	Yes
8X Concentra vs. 2X Concentra	0.0431	6	8.508	<0.001	Yes
8X Concentra vs. 1X Concentra	0.0373	6	7.363	<0.001	Yes
8X Concentra vs. 4X Concentra	0.0249	6	4.916	0.012	Yes
4X Concentra vs. Uncrosslinke	0.0418	6	8.252	<0.001	Yes
4X Concentration vs. Ethanol	0.0247	6	4.876	0.013	Yes
4X Concentra vs. 2X Concentra	0.0182	6	3.593	0.131	No
4X Concentra vs. 1X Concentra	0.0124	6	2.448	0.518	Do Not Test
1X Concentra vs. Uncrosslinke	0.0294	6	5.804	0.002	Yes
1X Concentration vs. Ethanol	0.0123	6	2.428	0.527	No
1X Concentra vs. 2X Concentra	0.00580	6	1.145	0.965	Do Not Test
2X Concentra vs. Uncrosslinke	0.0236	6	4.659	0.021	Yes
2X Concentration vs. Ethanol	0.00650	6	1.283	0.943	Do Not Test
Ethanol vs. Uncrosslinked	0.0171	6	3.376	0.179	No

A result of "Do Not Test" occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed means is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the means, even though one may appear to exist.

One Way Analysis of Variance--Energy to Break as a function of incubation time (Table 4.2)

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Data source: Data 1 in Notebook1

Group	N	Missing	Median	25%	75%
Uncrosslinked	10	0	0.0710	0.0315	0.176
Ethanol	10	0	3.033	1.765	4.190
30 minute incubation time (1X)	10	0	6.035	4.119	7.115
12 hour incubation time (1X)	10	0	3.869	3.243	5.251
24 hour incubation time (1X)	10	0	4.444	2.242	6.793

H = 28.368 with 4 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	p<0.05
30 minute inc vs Uncrosslinked	321.000	6.963	Yes
30 minute inc vs Ethanol	142.000	3.080	No
30 minute inc vs 12 hour incub	81.000	1.757	Do Not Test
30 minute inc vs 24 hour incub	61.000	1.323	Do Not Test
24 hour incub vs Uncrosslinked	260.000	5.640	Yes
24 hour incub vs Ethanol	81.000	1.757	Do Not Test
24 hour incub vs 12 hour incub	20.000	0.434	Do Not Test
12 hour incub vs Uncrosslinked	240.000	5.206	Yes
12 hour incub vs Ethanol	61.000	1.323	Do Not Test
Ethanol vs Uncrosslinked	179.000	3.883	Yes

Note: The multiple comparisons on ranks do not include an adjustment for ties.

A result of "Do Not Test" occurs for a comparison when no significant difference is found between the two rank sums that enclose that comparison. For example, if you had four rank sums sorted in order, and found no significant difference between rank sums 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed rank sums is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the rank sums, even though one may appear to exist.

One Way Analysis of Variance--Energy to Break as a function of Crosslinker concentration (Table 4.7)

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Passed (P = 0.133)

Equal Variance Test: Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Data source: Data 1 in Notebook1

Group	N	Missing	Median	25%	75%
Uncrosslinked	10	0	0.0710	0.0315	0.176
Ethanol	10	0	3.033	1.765	4.190
1X Concentration	10	0	6.035	4.119	7.115
2X Concentration	10	0	3.056	2.488	5.140
4X Concentration	10	0	4.282	3.404	5.025
8X Concentration	10	0	5.614	4.492	7.432

H = 34.428 with 5 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	p<0.05
8X Concentrat vs Uncrosslinked	397.000	7.189	Yes
8X Concentration vs Ethanol	193.000	3.495	No
8X Concentrat vs 2X Concentrat	165.000	2.988	Do Not Test
8X Concentrat vs 4X Concentrat	111.000	2.010	Do Not Test
8X Concentrat vs 1X Concentrat	16.000	0.290	Do Not Test
1X Concentrat vs Uncrosslinked	381.000	6.899	Yes
1X Concentration vs Ethanol	177.000	3.205	Do Not Test
1X Concentrat vs 2X Concentrat	149.000	2.698	Do Not Test
1X Concentrat vs 4X Concentrat	95.000	1.720	Do Not Test
4X Concentrat vs Uncrosslinked	286.000	5.179	Yes
4X Concentration vs Ethanol	82.000	1.485	Do Not Test
4X Concentrat vs 2X Concentrat	54.000	0.978	Do Not Test
2X Concentrat vs Uncrosslinked	232.000	4.201	Yes
2X Concentration vs Ethanol	28.000	0.507	Do Not Test
Ethanol vs Uncrosslinked	204.000	3.694	No

Note: The multiple comparisons on ranks do not include an adjustment for ties.

A result of "Do Not Test" occurs for a comparison when no significant difference is found between the two rank sums that enclose that comparison. For example, if you had four rank sums sorted in order, and found no significant difference between rank sums 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed rank sums is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the rank sums, even though one may appear to exist.

One Way Analysis of Variance--In Vitro Degradation DMEM as a function of incubation time (Figure 4.8)

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Passed (P = 0.480)

Equal Variance Test: Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Data source: Data 1 in Notebook1

Group	N	Missing	Median	25%	75%
30 minute incubation (1X)	9	0	90.400	83.910	100.000
12 hour incubation (1X)	9	0	75.810	71.975	81.635
24 hour incubation (1X)	9	0	84.170	82.930	86.630

H = 15.903 with 2 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	p<0.05
30 minute inc vs 12 hour incub	132.000	5.543	Yes
30 minute inc vs 24 hour incub	45.000	1.890	No
24 hour incub vs 12 hour incub	87.000	3.654	Yes

Note: The multiple comparisons on ranks do not include an adjustment for ties.

One Way Analysis of Variance--In Vitro Degradation DMEM as a function of Crosslinker concentration (Figure 4.18)

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Passed (P = 0.434)

Equal Variance Test: Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Data source: Data 1 in Notebook1

Group	N	Missing	Median	25%	75%
1X Concentration	9	0	90.400	83.910	100.000
2X Concentration	9	0	66.260	64.340	68.555
4X Concentration	9	0	42.510	41.435	46.605
8X Concentration	9	0	23.380	17.305	26.190

H = 32.855 with 3 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	p<0.05
1X Concentrat vs 8X Concentrat	243.000	7.688	Yes
1X Concentrat vs 4X Concentrat	162.000	5.125	Yes
1X Concentrat vs 2X Concentrat	81.000	2.563	No
2X Concentrat vs 8X Concentrat	162.000	5.125	Yes
2X Concentrat vs 4X Concentrat	81.000	2.563	No
4X Concentrat vs 8X Concentrat	81.000	2.563	No

Note: The multiple comparisons on ranks do not include an adjustment for ties.

One Way Analysis of Variance-In Vitro Degradation DMEM + 10% FBS as a function of incubation time (Figure 4.6)

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Passed (P = 0.067)

Equal Variance Test: Passed (P = 0.243)

Group Name	N	Missing	Mean	Std Dev	SEM
30 minute incubation time (1X)	9	0	100.000	0.000	0.000
12 hour incubation time (1X)	9	0	79.509	5.230	1.743
24 hour incubation time (1X)	9	0	79.513	6.413	2.138

Source of Variation	DF	SS	MS	F	P
Between Groups	2	2518.767	1259.384	55.166	<0.001
Residual	24	547.898	22.829		
Total	26	3066.666			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	p<0.050
30 minute in vs. 12 hour incu	20.491	3	12.866	<0.001	Yes
30 minute in vs. 24 hour incu	20.487	3	12.863	<0.001	Yes
24 hour incu vs. 12 hour incu	0.00444	3	0.00279	1.000	No

One Way Analysis of Variance-In Vitro Degradation DMEM + 10% FBS as a function of Crosslinker concentration (Figure 4.16)

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Data source: Data 1 in Notebook1

Group	N	Missing	Median	25%	75%
1X Concentration	9	0	100.000	100.000	100.000
2X Concentration	9	0	87.120	84.705	92.705
4X Concentration	9	0	59.810	52.130	62.550
8X Concentration	9	0	33.330	31.120	34.955

H = 32.846 with 3 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	p<0.05
1X Concentrat vs 8X Concentrat	238.500	7.546	Yes
1X Concentrat vs 4X Concentrat	157.500	4.983	Yes
1X Concentrat vs 2X Concentrat	72.000	2.278	No
2X Concentrat vs 8X Concentrat	166.500	5.268	Yes
2X Concentrat vs 4X Concentrat	85.500	2.705	No
4X Concentrat vs 8X Concentrat	81.000	2.563	No

Note: The multiple comparisons on ranks do not include an adjustment for ties.

One Way Analysis of Variance--In Vitro Degradation SSF as a function of incubation time (Figure 4.7)

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Data source: Data 1 in Notebook1

Group	N	Missing	Median	25%	75%
30 minute incubation time (1X)	9	0	100.000	100.000	100.000
12 hour incubation time (1X)	9	0	83.750	80.615	86.740
24 hour incubation time (1X)	9	0	83.740	81.490	86.475

H = 18.018 with 2 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	p<0.05
30 minute inc vs 24 hour incub	122.000	5.124	Yes
30 minute inc vs 12 hour incub	121.000	5.082	Yes
12 hour incub vs 24 hour incub	1.000	0.0420	No

Note: The multiple comparisons on ranks do not include an adjustment for ties.

One Way Analysis of Variance----In Vitro Degradation SSF as a function of Crosslinker concentration (Figure 4.17)

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Passed (P = 0.428)

Equal Variance Test: Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Data source: Data 1 in Notebook1

Group	N	Missing	Median	25%	75%
1X Concentration	9	0	100.000	100.000	100.000
2X Concentration	9	0	87.070	71.145	90.175
4X Concentration	9	0	79.860	74.470	85.250
8X Concentration	9	0	60.070	54.880	62.850

H = 30.132 with 3 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	p<0.05
1X Concentrat vs 8X Concentrat	243.000	7.688	Yes
1X Concentrat vs 4X Concentrat	129.000	4.081	Yes
1X Concentrat vs 2X Concentrat	114.000	3.607	No
2X Concentrat vs 8X Concentrat	129.000	4.081	Yes
2X Concentrat vs 4X Concentrat	15.000	0.475	No
4X Concentrat vs 8X Concentrat	114.000	3.607	No

Note: The multiple comparisons on ranks do not include an adjustment for ties.

One Way Analysis of Variance--Average Porosity as a function of incubation time (Figure 4.9)

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Passed (P = 0.132)

Equal Variance Test: Passed (P = 0.254)

Group Name	N	Missing	Mean	Std Dev	SEM
Uncrosslinked	5	0	82.148	4.099	1.833
Ethanol	5	0	84.578	5.261	2.353
30 minute incubation (1X)	5	0	67.804	3.677	1.644
12 hour incubation (1X)	5	0	37.116	6.468	2.893
24 hour incubation (1X)	5	0	64.118	6.425	2.873

Source of Variation	DF	SS	MS	F	P
Between Groups	4	7201.685	1800.421	63.795	<0.001
Residual	20	564.436	28.222		
Total	24	7766.121			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	p<0.050
Ethanol vs. 12 hour incu	47.462	5	19.977	<0.001	Yes
Ethanol vs. 24 hour incu	20.460	5	8.612	<0.001	Yes
Ethanol vs. 30 minute in	16.774	5	7.060	<0.001	Yes
Ethanol vs. Uncrosslinked	2.430	5	1.023	0.949	No
Uncrosslinke vs. 12 hour incu	45.032	5	18.955	<0.001	Yes
Uncrosslinke vs. 24 hour incu	18.030	5	7.589	<0.001	Yes
Uncrosslinke vs. 30 minute in	14.344	5	6.038	0.003	Yes
30 minute in vs. 12 hour incu	30.688	5	12.917	<0.001	Yes
30 minute in vs. 24 hour incu	3.686	5	1.551	0.806	No
24 hour incu vs. 12 hour incu	27.002	5	11.366	<0.001	Yes

One Way Analysis of Variance--Average Porosity as a function of Crosslinker concentration (Figure 4.19)

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Passed (P = 0.460)

Equal Variance Test: Passed (P = 0.276)

Group Name	N	Missing	Mean	Std Dev	SEM
Uncrosslinked	5	0	82.148	4.099	1.833
Ethanol	5	0	84.578	5.261	2.353
1X Concentration	5	0	67.804	3.677	1.644
2X Concentration	5	0	54.574	6.467	2.892
4X Concentration	5	0	36.978	10.521	4.705
8X Concentration	5	0	54.770	8.895	3.978

Source of Variation	DF	SS	MS	F	P
Between Groups	5	8349.268	1669.854	34.593	<0.001
Residual	24	1158.529	48.272		
Total	29	9507.797			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	p<0.050
Ethanol vs. 4X Concentration	47.600	6	15.319	<0.001	Yes
Ethanol vs. 2X Concentration	30.004	6	9.656	<0.001	Yes
Ethanol vs. 8X Concentration	29.808	6	9.593	<0.001	Yes
Ethanol vs. 1X Concentration	16.774	6	5.399	0.010	Yes
Ethanol vs. Uncrosslinked	2.430	6	0.782	0.993	No
Uncrosslinke vs. 4X Concentra	45.170	6	14.537	<0.001	Yes
Uncrosslinke vs. 2X Concentra	27.574	6	8.874	<0.001	Yes
Uncrosslinke vs. 8X Concentra	27.378	6	8.811	<0.001	Yes
Uncrosslinke vs. 1X Concentra	14.344	6	4.616	0.034	Yes
1X Concentra vs. 4X Concentra	30.826	6	9.921	<0.001	Yes
1X Concentra vs. 2X Concentra	13.230	6	4.258	0.060	No
1X Concentra vs. 8X Concentra	13.034	6	4.195	0.065	Do Not Test
8X Concentra vs. 4X Concentra	17.792	6	5.726	0.006	Yes
8X Concentra vs. 2X Concentra	0.196	6	0.0631	1.000	Do Not Test
2X Concentra vs. 4X Concentra	17.596	6	5.663	0.006	Yes

A result of "Do Not Test" occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed means is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the means, even though one may appear to exist.

One Way Analysis of Variance--Swelling as a function of incubation time

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Data source: Data 1 in Notebook1

Group	N	Missing	Median	25%	75%
Uncrosslinked	35	0	662.774	562.774	728.161
Ethanol	35	0	435.185	357.870	487.500
30 minute incubation (1X)	35	0	470.023	428.571	518.211
12 hour incubation (1X)	35	0	220.521	194.005	261.889
24 hour incubation (1X)	35	0	261.887	201.071	432.619

H = 74.112 with 4 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	p<0.05
Uncrosslinked vs 12 hour incub	3314.000	11.057	Yes
Uncrosslinked vs 24 hour incub	2686.000	8.962	Yes
Uncrosslinked vs Ethanol	1620.000	5.405	Yes
Uncrosslinked vs 30 minute inc	1260.000	4.204	Yes
30 minute inc vs 12 hour incub	2054.000	6.853	Yes
30 minute inc vs 24 hour incub	1426.000	4.758	Yes
30 minute inc vs Ethanol	360.000	1.201	No
Ethanol vs 12 hour incub	1694.000	5.652	Yes
Ethanol vs 24 hour incub	1066.000	3.557	No
24 hour incub vs 12 hour incub	628.000	2.095	No

Note: The multiple comparisons on ranks do not include an adjustment for ties.

One Way Analysis of Variance--Swelling as a function of Crosslinker concentration (Figure 4.20)

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Data source: Data 1 in Notebook1

Group	N	Missing	Median	25%	75%
Uncrosslinked	35	0	662.774	562.774	728.161
Ethanol	35	0	435.185	357.870	487.500
1X Concentration	35	0	470.023	428.571	518.211
2X Concentration	35	0	384.071	357.227	406.733
4X Concentration	35	0	201.556	187.568	210.860
8X Concentration	35	0	123.895	110.773	129.951

H = 103.116 with 5 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	p<0.05
Uncrosslinked vs 8X Concentrat	4476.000	12.451	Yes
Uncrosslinked vs 4X Concentrat	3576.000	9.947	Yes
Uncrosslinked vs 2X Concentrat	2469.000	6.868	Yes
Uncrosslinked vs Ethanol	1619.000	4.504	Yes
Uncrosslinked vs 1X Concentrat	1216.000	3.383	No
1X Concentrat vs 8X Concentrat	3260.000	9.068	Yes
1X Concentrat vs 4X Concentrat	2360.000	6.565	Yes
1X Concentrat vs 2X Concentrat	1253.000	3.485	No
1X Concentration vs Ethanol	403.000	1.121	Do Not Test
Ethanol vs 8X Concentration	2857.000	7.947	Yes
Ethanol vs 4X Concentration	1957.000	5.444	Yes
Ethanol vs 2X Concentration	850.000	2.364	Do Not Test
2X Concentrat vs 8X Concentrat	2007.000	5.583	Yes
2X Concentrat vs 4X Concentrat	1107.000	3.079	No
4X Concentrat vs 8X Concentrat	900.000	2.504	No

Note: The multiple comparisons on ranks do not include an adjustment for ties.

A result of "Do Not Test" occurs for a comparison when no significant difference is found between the two rank sums that enclose that comparison. For example, if you had four rank sums sorted in order, and found no significant difference between rank sums 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed rank sums is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the rank sums, even though one may appear to exist.

One Way Analysis of Variance—Mucoadhesion (Figure 4.21)

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Passed (P = 0.618)

Equal Variance Test: Passed (P = 0.157)

Group Name	N	Missing	Mean	Std Dev	SEM
4X Nanofiber	8	0	0.262	0.0775	0.0274
8X Nanofiber	8	0	-0.134	0.106	0.0375
4X Hydrogel	8	0	-0.0790	0.0889	0.0314
PEG-1500 Hydrogel	8	0	-0.0850	0.0368	0.0130

Source of Variation	DF	SS	MS	F	P
Between Groups	3	0.797	0.266	40.062	<0.001
Residual	28	0.186	0.00663		
Total	31	0.982			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Tukey Test):
Comparisons for factor:

Comparison	Diff of Means	p	q	P	p<0.050
4X Nanofiber vs. 8X Nanofiber	0.396	4	13.752	<0.001	Yes
4X Nanofiber vs. PEG-1500 Hyd	0.347	4	12.041	<0.001	Yes
4X Nanofiber vs. 4X Hydrogel	0.341	4	11.832	<0.001	Yes
4X Hydrogel vs. 8X Nanofiber	0.0553	4	1.919	0.536	No
4X Hydrogel vs. PEG-1500 Hyd	0.00600	4	0.208	0.999	Do Not Test
PEG-1500 Hyd vs. 8X Nanofiber	0.0493	4	1.711	0.626	Do Not Test

A result of "Do Not Test" occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed means is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the means, even though one may appear to exist.

One Way Analysis of Variance--Drug Release Kinetics (Figure 4.22)

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks

Data source: Data 1 in Notebook1

Group	N	Missing	Median	25%	75%
50 mg dosage	30	0	0.448	0.256	0.729
100 mg dosage	30	0	1.135	0.498	1.910
200 mg dosage	30	0	1.234	0.518	2.146
400 mg dosage	30	0	1.810	0.753	3.302

H = 31.379 with 3 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	p<0.05
400 mg dosage vs 50 mg dosage	1485.000	7.794	Yes
400 mg dosage vs 100 mg dosage	783.000	4.110	Yes
400 mg dosage vs 200 mg dosage	540.000	2.834	No
200 mg dosage vs 50 mg dosage	945.000	4.960	Yes
200 mg dosage vs 100 mg dosage	243.000	1.275	No
100 mg dosage vs 50 mg dosage	702.000	3.685	Yes

Note: The multiple comparisons on ranks do not include an adjustment for ties.

VITA

Donald C. Aduba, Jr. was born in Maryville, Missouri on January 28, 1986 to Nigerian immigrant parents. He was raised in Kansas City, Missouri and lived there until graduating from Rockhurst High School and matriculating at the University of Virginia in Fall 2004. He graduated from UVa with a Bachelor of Science in Sports Medicine with a minor in Biomedical Engineering in August 2008. During his time at UVa, Donald participated in the National Society of Black Engineers, The Impact Movement, UVa Intramurals-Recreation Sports, Madison House charities and worked at Newcomb Student union. He also joined the Zeta Eta undergraduate chapter of Phi Beta Sigma Fraternity, Incorporated in April 2008. After graduating from college, Donald worked at Atlantic Coast Athletic Club as a Youth Counselor and volunteered designing artificial extracellular matrix systems at the University of Virginia Department of Regenerative Medicine laboratory under Dr. Roy Ogle. Donald began his graduate studies at Virginia Commonwealth University's Biomedical Engineering program in Fall 2009. During his time as a Master's student, Donald has obtained a multitude of great experiences as a student, researcher and teacher having been afforded opportunities to take informative classes, teach Pre-calculus to incoming undergraduate students and work as a research assistant in the Yang department of Biomaterials and Drug Delivery. He hopes to continue his growth as a doctoral student to make significant contributions at Dr. Yang's lab, VCU and the greater scientific community.